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New Editors of

Acta Pharmacologica et Toxicologica.

With this issue Professor Knud Møller ceases to be Editor in-chief of *Acta Pharmacologica et Toxicologica*. His friends and co-editors have tried to persuade him to continue his admirable editorial work for some years more, but in vain he is firm in his decision to retire.

Professor Møller was one of the founders of this Journal, indeed, he was undoubtedly the principal founder Moreover without his enthusiasm the Journal would never have become a reality From the very first issue, which by a curious coincidence appeared exactly on the day on which the Second World War was over for Denmark, and until now *Acta Pharmacologica et Toxicologica* has been characterized by his scientific enthusiasm and scrupulous editing. But, more than that, Møller s passion for system and consistency gave the Journal some of its main characteristics. He it was who worked out the rules for lay-out, arrangement, tables, figures, references, names, abbreviations, with a host of other items still more important, he it was who has seen them rigorously followed in every one of some 800 papers that have so far appeared in it. To some authors these rules may seem to be rather pedantic trivialities, but for the busy reader they are in fact important facilities, something that even the authors occasionally acknowledge when they come to see their articles in print.

Professor Dalgaard Mikkelsen, of the Royal Danish Veterinary School, and Dr Jens Schou, Møller s senior assistant at the Department of Pharmacology of the University of Copenhagen, will jointly take on what has been Møller s editorial work. Their task will be the easier in that the road has been paved by the practice evolved during nearly two decades under Møller's direction. It is some consolation that Møller is still to be a member of the editorial staff and is still within reach for the new Editors-in-chief whom he has promised to help on all points of doubt.

All editors of Acta will surely wish to express to Møller their warmest thanks for what he has done on behalf of *Acta Pharmacologica et Toxicologica* and the hope that we will be able for long to continue his editorial work for the Journal of his own creation.

13/1–64 – *Erik Jacobsen*

cess. Accordingly treatment with antirheumatic drugs or pathophysiological conditions known to inhibit oedema formation may exert this influence, primarily by altering the chemical composition of the oedema.

For the proper study of these questions, knowledge of the variations, if any occurring during the natural course of the inflammatory reaction is required. In our study the concentrations of fatfree solids, hexosamine, hydroxyproline, sodium, chloride and potassium in the oedema were determined at different intervals after application of the irritant.

Methods

White male mice of the Leo strain were used, weighing from 22 to 28 g and maintained on a standard laboratory diet and unrestricted water

The experimental procedures were recently described in detail (SZPORNY LANGGÅRD & HVIDBERG 1964). Briefly the procedure is that the back of the animal was treated on the day before the experiment with a depilatory containing barium sulphide the animal is put under light halothane, B.P (fluothane ®), anaesthesia, and two symmetrical areas were marked out on its skin. Acute inflammation was provoked within the borders of one of the areas by applying xylol for two minutes, with the contra-lateral area serving as control.

Five and 15 minutes, and 1 2, 3 6 18 24 and 48 hours later groups of animals were killed and bled. From each animal two skin samples were excised along the outlines of the areas, and the weight of the oedema was determined as the difference in weight of the two samples. Samples were dried, defatted and subsequently analysed. The analytical values for the oedema were calculated by the formula given in the introduction.

Hydroxyproline, hexosamine, sodium, chloride and potassium determinations were carried out as described by LANGGÅRD JENSEN-HOLM & HVIDBERG (1963).

Results

The average total weights of oedematous and corresponding non oedematous skin samples in relation to the duration of oedema formation are shown in fig. 1a (0–2 hours) and fig. 1b (1–48 hours) by dotted lines. The full line indicates the amount (= weight) of the oedema. The standard deviations are given by vertical lines through the points.

It appears that the weights of the non-oedematous side have a tendency to follow those of the oedematous side, although the ranges are much smaller This "resonance" was considered to result from a slight seeping of xylol from the treated area into the control area, rather than from any humoral mechanisms.

The curves illustrate the fact that oedema formation occurs in two steps. There is an immediate rise in oedema weight to a maximum two hours after application of the xylol, succeeded by a gradual decrease

in

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From the Department of Pharmacology University of Copenhagen
(Professor K O Møller M D)

The Chemical Composition of the Oedema Fluid during the Course of Acute Inflammation

By

H. Langgård, E. Hvidberg and L. Szporny[1])

(Received November 19 1963)

In a recent paper we have described a new method for quantitative investigations on acute inflammatory oedema in mice (SZPORNY *et al* 1964) It was based on the practicability of cutting out from untreated animals, two skin samples, of negligible weight difference, marked out symmetrically by a stamp on the depilated skin. When acute inflammation was provoked within the borders of one of the two areas, the other serving as control, the size of the oedema was defined as the weight difference between the two samples ($w_1 - w_2$) The total amounts of any given constituent were determined in the oedematous skin sample (a_1) and in the control sample (a_2) and the concentration of that constituent (c_e) was calculated by the formula $c_e\,\% = \frac{a_1 - a_2}{w_1 - w_2}\,100$

Thus "oedema in our terminology refers to a weight difference This concept of oedema permits quantitative calculations of the composition of oedema fluid at the site of formation in the connective tissue. Usually the term "oedema" refers to an abnormal accumulation of fluid in the connective tissue, *i e* to a mixture of exudation and pre-existing extra cellular fluid or simply to the oedematous state of the tissue. In contrast to this we refer to the excess fluid or genuine oedema *i e* the plasma filtrate.

In the report mentioned above it was demonstrated that 15 minutes after initiation of the inflammatory process the oedema had the same composition as blood plasma.

However during an acute inflammatory process changes in chemical composition of the oedema may interfere with the later course of the pro

1) Research fellow of the International Atomic Energy Agency

Table 1

Concentration of fat free solids, hexosamine, sodium and chloride in oedema fluid at various times during its formation, compared with plasma concentrations.

	Plasma	*Oedema*					
	(= 23) mean s.e.m.	5 min. (= 5) mean s.e.m.	15 min. (= 16) mean s.e.m.	1 h. (= 5) mean s.e.m.	2 h. (= 10) mean s.e.m.	6 h. (= 5) mean s.e.m.	48 h. (= 10) mean s.e.m.
Fat-free solids (%)	8.3[1]	7.6 ± 0.70	8.5 ± 0.51	5.6 ± 0.55	6.8 ± 0.45	6.3 ± 0.62	11.7 ± 1.07
Hexosamine (mg/100 g)	95[2]	89 ± 13.5	99 ± 7.9	85 ± 15.8	64 ± 5.3	65 ± 6.8	177 ± 11.9
Sodium (mEq/l)	136 ± 3.5	133 ± 11.4	144 ± 5.0	125 ± 6.2	123 ± 7.0	129 ± 7.1	132 ± 19.7
Chloride (mEq/l)	105 ± 1.2	111 ± 6.8	110 ± 5.7	111 ± 6.9	90 ± 4.0	103 ± 10.7	88 ± 6.9

1) From the literature.
2) = 5.

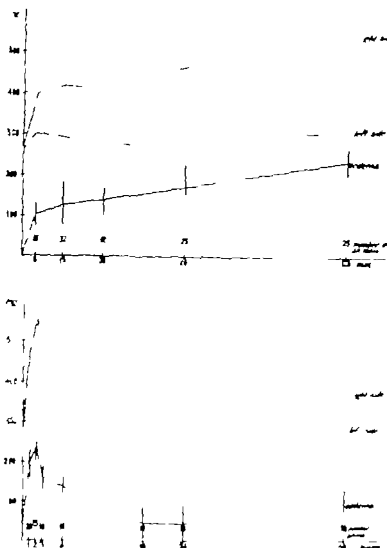


Fig. 1 Weight of oedema fluid during its two phases (0–2 hours fig. 1 a 1–48 hours fig. 1 b), determined as the differences between the treated and the untreated side plotted against time. Dotted curves represent total weights. Values for the standard deviations are indicated by vertical lines through the points.

during the following 18–24 hours By this time a second phase of oedema formation has already begun

The concentrations of fat free solids, hexosamine, sodium and chloride in plasma and in oedema fluid 15 minutes and 1 2, 6 and 48 hours after induction of the oedema are summarized in table 1 These figures demonstrate that the oedema at all hours up to and including 6 hours has the same composition as plasma. At 48 hours the concentrations of sodium and chloride are still the same as those in plasma, but there is a highly significant increase in the concentrations of fat free solids and hexosamine.

Table 3

Composition of inflammatory oedema fluid 24 hours after application of xylol. When oedema was absorbed, the figures indicate the difference between treated and untreated sides.

N	Total oedema (mg)	Solids mg	Solids percent	Sodium total µeq	Sodium meq/l	Chloride total µeq	Chloride meq/l	Hexosamine total µg	Hexosamine mg/kg	Potassium (µeq)	Hydroxyproline (mg)
1	165	19	11.5	23.8	156	21.4	129	212	128	3.4	−0.30
2	152	12	8.0	21.3	134	17.0	112	103	68	1.7	0.22
3	78	11	14.0	5.5	70	7.6	98	148	189	0.8	1.13
4	70	4	5.7	19.3	273	9.3	132	183	262	0.8	0.12
5	67	9	14.5	8.5	126	6.8	94	94	140	0.8	0.28
6	48	6	12.5	4.4	91	5.7	118	30	63	1.0	0.84
7	48	5	9.6	4.9	103	3.8	79	50	103	1.9	−0.07
			(av 10.8)	–	(av 137)	–	(av 109)	–	(av 127)		–
8	27	3		6.9	–	1.8		17	–	−2.2	−0.12
9	24	0		3.8	–	2.1		42	–	−1.0	−0.10
10	21	2		2.8		1.5		43	–	−0.4	0.09
11	12	1		16.6		5.6	–	34	–	−0.3	−0.20
12	7	2		2.0		1.4		56	–	−0.3	0.27
13	0	0		0.9	–	1.5	–	34		−2.2	−0.03
14	−4	−3		5.5		1.0	–	10		−0.6	−1.41
15	−12	−6		0.2	–	1.0	–	27	–	−1.6	−0.08
16	−15	−4		1.8	–	0.2		84	–	−1.8	−0.29
17	−15	−4		−0.4	–	−1.3		13	–	−1.8	−0.12

Table 2 shows the total amounts of hydroxyproline and potassium in oedematous and non-oedematous skin and in the oedema fluid itself. At no time does the oedema contain significant amounts of hydroxyproline. The contents of potassium are negligible until 6 hours and at 48 hours amount to 4.0 µeq.

Because of the variable results obtained on the 24 hours group of mice these values are given in a separate table. In table 3 the animals are listed individually in descending order of weight of oedema.

One animal (no. 13) had no oedema, and in four animals (no. 14–17) the treated side weighed less than the untreated side.

The hydroxyproline contents were the same on both sides.

Seven animals (no. 1–7) with over 30 mg of oedema (previously shown to be a limit below which concentrations cannot be calculated with sufficient accuracy) showed the average values for the concentrations of sodium and chloride to be the same as in the earlier groups. The concentrations of hexosamine and solids, however, were slightly higher.

Ten animals (no. 8–17) with little or no oedema constantly showed less potassium on the treated side than on the corresponding untreated side, although there was a clear excess of sodium, chloride and hexosamine on the treated side.

Table 2

Tot l amount of potassium and hydroxyproline in inflamed skin in the control samples and in the oedema fluid at various times during oedema formation.

		5 min. (n = 5) mean s.e.m.	15 min. (n = 16) mean s.e.m.	1 h. (n = 5) mean s.e.m.	2 h. (n = 10) mean s.e.m.	6 h. (n = 5) mean s.e.m.	48 h. (n = 10) mean s.e.m.
Total potassium (µEq)	treated side	11.3	12.6	8 9	12.8	12.1	16 0
	untreated side	10 7	12.5	10.4	13 1	12.3	12.0
	oedema	0 6 ± 0 41	0.1 ± 0.16	−1 5 ± 0.47	−0 3 ± 0.38	−0.2 ± 0 38	4 0 ± 0.50
Total hydroxyprol ne (mg)	treated side	3 83	4.23	3 72	4 63	3 36	3 79
	untreated side	3 83	3 79	3 48	4 45	3 33	3 46
	oedema	0.00 ± 0 10	0.44 ± 0.14	0.24 ± 0.17	0.18 ± 0 13	0.03 ± 0 06	0.25 ± 0.10

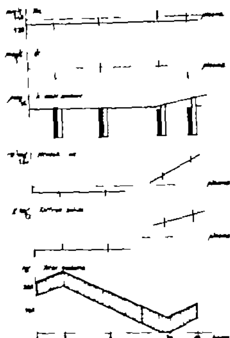


Fig. 2. Weight and composition of oedema fluid in relation to time (log scale). The dotted lines represent corresponding plasma concentrations of sodium, chloride, hexosamine and fat-free solids. The standard errors of the means are indicated by vertical lines. Because of the small amounts of potassium present, the figures for the total amounts of potassium in inflamed skin samples (dark columns) and control samples (plain columns) are shown instead.

nate (KULONEN 1952 HVIDBERG, LANGGÅRD & SZPORNY 1964) since its formation would explain the concomitant rise in the potassium contents (tables 1 and 2) The high concentration of fat free solids in the late oedema fluid must have been caused by an accumulation of proteins other than collagen, since the amounts of hydroxyproline remained unchanged (table 2). It is noteworthy that the amounts of collagen were not altered at this stage of the regenerative phase (cf HOUCK & JAKOB 1961)

In fig. 2 an attempt is made to illustrate more schematically the findings of our study The concentrations of fat-free solids, hexosamine, sodium and chloride, the total amounts of potassium of oedematous skin with corresponding control values and the "oedema weight" have been related to time (1–48 hours, on a logarithmic scale).

It can be stated in conclusion that two distinct phases occur during the acute inflammatory process provoked by the procedure we have applied. In these two phases the type of fluid accumulation is different. The initial oedema fluid throughout its formation and reabsorption maintains the

Discussion

The two-phased course of the curve in fig. 1 b indicates that the early oedematous stage of the inflammatory process does not pass directly into the oedematous state of the regenerative phase Approximately 24 hours after induction of the inflammatory reaction the weight of the oedema has decreased to a minimum and thereafter another oedematous state is developed. The fact that all of the animals in the 48 hour group had unequivocal oedema, although several animals in the 18- and 24 hour groups showed none, suggests that the oedema of each animal at somewhere about 18–24 hours is in fact reduced to zero

Throughout the initial phase of the process the inflammatory oedema fluid has a constant composition, the same as that of plasma (tables 1 and 3) Although the principal route of protein absorption is presumably via the lymphatics, whereas water and crystalloids mainly are absorbed directly into the blood stream (HOLLANDER *et al* 1961 LANGGÅRD 1963), the concentration of fat free solids in the oedema remains unchanged during the absorption phase. This is even more surprising because the term oedema in the present study refers to the amounts of fluid in fact filtrated through the capillary membrane (see above) and not to the mixture that the plasma filtrate in all probability forms with the pre-existing extracellular fluid In other words, there are some indications that a certain fraction of this total oedema is kept in equilibrium with the plasma throughout the formation and reabsorption of the oedema fluid.

At the transitory stage 24 hours after application of xylol highly heterogenous results were obtained (table 3) The figures seem to indicate that some of the animals at this time were in the late part of the first phase, while others were in the early part of the second phase As stated above the accuracy of the calculations was also less in this group because of the small size of the oedemas. It seems significant however that at this intermediate stage of the inflammatory process a shifting of electrolytes is taking place Animals with little or no oedema show a loss of potassium on the inflamed side with a small but clear excess of sodium and chloride. It is also to be noted that the amounts of hexosamine have increased. For the study of connective tissue as an organ" depositing water and electrolytes these findings may be of importance Extended work with the specific object of clarifying questions related to the transitory phase should be carried out before conclusions are drawn.

In the late phase of the acute inflammatory process a production of acid mucopolysaccharides, presumably hyaluronic acid (WHITE, SHETLAR and SCHILLING 1961) has taken place. At the existing tissue pH hyaluronic acid primarily occurs as a salt, most likely as potassium hyaluro-

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Salivary Excretion of Sulphonamides and Barbiturates by Cows and Goats

By

Folke Rasmussen

(Received November 22, 1963)

KILLMANN & THAYSEN (1955) demonstrated that various sulphonamides were excreted by man in the parotid saliva. The concentration in this acid saliva was found to be lower than that in plasma, and the ratio between the saliva and the plasma concentrations was correlated with the pK_a-value of the sulphonamide in question, as only the un-ionized form of the drug diffused across the epithelium of the salivary gland. Investigating the excretion in other acid secretions, THAYSEN & SCHWARTZ (1953) observed that sulphonamide excretion in sweat similarly depended on the physico-chemical properties of the drugs. The same fact was demonstrated in studies of mammary excretion of *e. g.* sulphonamides, penicillin, penethamate, erythromycine, antipyrine (RASMUSSEN 1958, 1959, 1961) and barbiturates (unpublished results).

As the parotid secretion in ruminants is alkaline, drug excretion in cow and goat saliva should offer an opportunity for additional appraisal of the correlation between ionization and diffusion of drugs. Previous findings indicate that acid drugs should be excreted in this alkaline secretion at higher concentrations than those obtaining in ultrafiltrates of plasma.

Materials and Methods

Five cows and eleven goats were used. Mixed saliva from the cows was collected by means of a gauze pad, whereas in goats parotid saliva was collected continuously through a plastic catheter inserted in the parotid duct. The sulphonamides were injected intravenously into cows over five minutes and administered to goats by permanent infusion through a plastic catheter (ROBERTS & DYE 1951) in the left jugular

composition of a bulk filtrate of plasma, whereas the later phase is characterized by higher concentrations of fat free solids, hexosamine and *potassium indicating profound alterations in* the connective tissue ground substance

Summary

The quantity and chemical composition of inflammatory oedema fluid *has been followed through the natural course of an experimentally induced* acute inflammatory process The oedema was examined *in situ* and control studies were carried out on the same animals

It was found that oedema formation occurred in two phases The initial phase, during which the oedema maintained the composition of a bulk filtrate of plasma lasted approximately 24 hours The second phase, which had probably been initiated before the end of this period, was characterized by a marked increase in the amounts of fat free solids, hexosamine and potassium indicating changes in the connective tissue ground substance characteristic of regenerative processes. At the transitory stage 24 hours after initiation of the oedema excessive amounts of sodium and chloride but a deficit of potassium were noted in the inflamed tissues This point needs further clarification.

Acknowledgements

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vein. The barbiturates were injected intravenously in separate doses, initially 20-200 mg/kg and smaller supplementary doses at intervals Sampling was begun 30-60 minutes after beginning injection. Blood samples were drawn through a permanent cannula in the right jugular vein. In each experiment six samples of blood and saliva were drawn at intervals of about 15 minutes Immediately after each sampling, the pH values of the blood and the saliva were measured potentiometrically with a microglass electrode at 37°C. The content of unacetylated sulphonamide in the samples was determined by the method of BRATTON & MARSHALL (1939) and barbiturate determination was performed by the method of LOUS (1950) Protein-binding was determined by the ultrafiltration method of POULSEN (1956) Lipid solubility was expressed as the chloroform/water molecular distribution of each drug.

Results

The concentration ratio saliva/ultrafiltrate of plasma

After administration of sulphanilamide sulphadimidine or sulphaethoxypyridazine at a dosage of 100 mg/kg body weight to five cows and one goat, the concentrations of unacetylated sulphonamide were determined in all simultaneously drawn saliva and plasma samples and in the ultrafiltrate of the plasma. The results from these preliminary experiments at falling plasma concentrations are summarized in table 1 where it is seen that the ratio between the sulphanilamide concentrations in saliva and the ultrafiltrate of plasma was about 1 0 whereas the concentration of sulphadimidine and sulphaethoxypyridazine was higher in saliva than in ultrafiltrate.

Table 2 records a list of the test substances and the experimental results, computed as average concentrations from six observations in each of 21 experiments on nine goats at approximately constant plasma concentra

Table 1

Concentrations of sulphonamides in plasma ultrafiltrate of plasma a d saliva at falling plasma concentrations.

	Observa tions no	Plasma µg/ml	Ultrafil trate of plasma µg/ml	Sal va µg/ml	R tio S P	Ult
Sulphanilamide	7	6 95	53–81	52–80		1 0
Sulphadimidine	18	32 [illegible]36	8 1[illegible]3	16– 58	1 3	3 3
Sulphaethoxypyridazine	2[illegible]	54–333	4–67	[illegible] 139	1 3	2.4

tions. The ratio between the concentrations in saliva and plasma (S/P) is 0.7 or higher except for those of sulphacetamide. After correction for plasma protein binding, the corresponding ratio (S/P Ultr) is 1.0 for sulphanilamide and still extremely low for sulphacetamide, whereas the ratios for sulphadimidine, sulphadiazine, and the four barbiturates are higher than 1.0 and maximally 3.8. None of the drugs examined was protein bound in saliva.

The difference in magnitude of the ratios is also evident from fig. 1 which is a record of experiments with sulphanilamide, sulphadimidine and phenobarbitone. The rate of secretion varied from 0.04–2.4 ml/min.

Table 2

Concentration of sulphonamides and barbiturates in plasma and parotid saliva and in the ultrafiltrates of plasma from goats.

	Plasma µg/ml	Saliva µg/ml	Ratio S/P	Protein-binding	Ultrafiltrat of plasma µg/ml	Ratio S/P Ultr
Sulphanilamide	121	93	0.8	22	94	1.0
	27	21	0.8	[illegible]	21	1.0
Sulphadimidine	236	202	0.9	57	102	2.0
	102	77	0.8	64	37	2.1
Sulphadiazine	266	233	0.9	22	208	1.1
	252	274	1.1	20	201	1.4
	213	504	2.4	3	145	3.5
Sulphacetamide	80	9	0.1	21	63	0.1
	27	3	0.1	37	17	0.2
Pentobarbitone (Mebumalum)	115	115	1.0	67	38	3.0
	55	40	0.7		18	2.2
	51	64	1.3	66	17	3.8
	51	36	0.7		17	2.1
Barbitone (Diemalum)	269	570	2.1	11	239	2.4
	13	279	1.3	21	168	1.7
	61	80	1.3	50	31	[illegible]
Reposal ®[1])	90	98	1.1	69	28	3.5
	51	88	1.7	55	23	3.8
Phenobarbitone (Phenemalum)	107	123	1.[illegible]	68	34	3.6
	86	98	1.1	68	28	3.5
	28	32	1.1	58	12	2.7

[1]) Reposal ®: 5-(bicyclo-3,2,1-oct-2-en-yl)-5-ethylbarbituric acid. Generously supplied by Novo Terapeutisk Laboratorium A/S, Copenhagen.

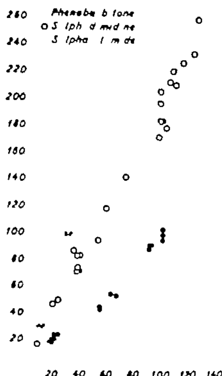


Fig. 1 Relation between the concentrations of sulphanilamide, sulphadimidine and phenobarbitone in saliva and the ultrafiltrates of plasma
Ordinate Concentration in saliva µg/ml.
Abscissa Concentration in the ultrafiltrate of plasma µg/ml.

in these experimental periods The figure shows that the salivary concentration is directly proportional to the concentration in the ultrafiltrate of plasma and that the ratios are different for the three compounds

The influence of secretion rate on the concentration ratio saliva/ultrafiltrate of plasma

In the experiments on goats the parotid secretion rate varied from 0 04 to 7 0 ml/min. and is recorded in table 3 together with the experimentally established ratios The ratios include only periods in which the salivary pH was 8.2–8 4 so that the influence of the greater pH variations in the saliva is eliminated The table shows that the ratio S/P Ultr for each drug was constant and independent of variations in the secretion rate up to 2–3 ml/min., whereas it may appear to diminish at high secretion rates (5–7 ml/min.)

Table 3

Ratio of saliva to plasma ultrafiltrate concentrations and variations in secretory intensity in goats.

	Ratio S/P Ultr	Saliva ml/min.		Ratio S/P Ultr	Saliva ml/min.
Sulphanilamide	1.1	0.5	Pentobarbitone	2.6	1.0
	1.1	0.6		2.2	[illegible]
	1.0	0.7		2.5	2.2
	1.0	0.7		2.2	2.3
	1.0	1.0		2.1	3.0)
	1.0	1.0		1.9	5.5
Sulphadimidine	2.2	0.4	Barbitone	[illegible]	0.2
	2.1	0.7		2.6	0.2
	1.9	0.7		2.5	0.2
	2.1	0.9		2.3	0.6[1])
	1.9	1.7[1])		2.5	1.2
	1.9	2.2		2.7	1.6
Sulphadiazine	1.3	0.4	Phenobarbitone	3.7	0.3
	0.9	0.6		3.6	0.5
	1.4	3.3		3.6	0.6
	1.2	4.6		2.6	1.4)
	1.1	5.5		3.4	1.4
	1.0	7.0[1])		3.6	2.4

[1]) 10 min. before this sample 0.5 ml inject. carbacholin. was injected i.m.

The degree of ionization compared to the concentration ratio saliva/ultrafiltrate of plasma.

The pK_a values for each drug are given in table 4. By means of the formula (*cf.* Albert 1952)

$$\text{percentage un-ionized} = 100 - \frac{100}{1 + \text{antilog}\,(pK_a - pH)}$$

the percentage of un-ionized drug was calculated at the pH of blood and saliva. The pH of the saliva samples varied from 8.0 to 8.4, the pH of the blood samples was 7.3–7.5 and in one experiment 7.1. The results are recorded in columns 3 and 4 of table 4. If only the non-protein bound and un-ionized fraction of drugs are able to diffuse across the salivary epithelium and establish equilibrium, the formula

$$R = \frac{1 + 10^{(pH_s - pK_a)}}{1 + 10^{(pH_p - pK_a)}}$$

permits calculation of this ratio when equilibrium is established (*cf. e.g.* Jacobs 1940, Davson & Danielli 1943). In the formula pH$_s$ denotes

Table 4

Comparison of found and calculated ratios

	pKa	Un-ionized in plasma pH 7.4	Un-ionized in saliva pH 8	Calculated S/P Ultr	Found S/P Ultr
Sulphanilamide	10.4	100	100	1.0	1.0
Pentobarbitone	8.0	80	39	2.1	[illegible]
Barbitone	7.8	7	4[1]	3.0	2.2
Reposal ®	7.5	61[2]	17	3.6	3.7
Sulphadimidine	7.4	50	14	3.6	[illegible]
Phenobarbitone	7	39	9	4.3	3.3
Sulphadiazine	6.5	11		5.5	1.3
		70[3]		10	3.5
Sulphacetamide	5.4	1	0.1	10	0.2

1) pH 8.3
2) pH 7.3
3) pH 7.1

the pH of the secretion and pH_p the pH of the blood. Theoretical ratios calculated by this formula are given in table 4 and next to them the average values of the ratios found experimentally. The table shows a ratio of 1.0 for sulphanilamide, which exists exclusively in un-ionized form both in saliva and in blood. The remaining drugs are present both un-ionized and ionized in blood, as in saliva, but the un-ionized fraction is larger in blood than in saliva. Accordingly, an equilibration of the un-ionized fractions on each side of the membrane will result in the appearance of a larger total quantity of the drug in saliva than in the ultrafiltrate of plasma. In support of this theory, the experimental results recorded in table 4 show that all these drugs except sulphacetamide were concentrated in the saliva.

Discussion

Several studies, reviewed by SCHANKER (1962), have demonstrated that the distribution of a large number of drugs across membranes in the animal organism depends on the pK value of the drug and the pH difference between the biological fluids on either side of the membrane. In our investigation of the salivary excretion of two homologous drug series in ruminants, this phenomenon was further illustrated. Except for sulphacetamide, these weakly acid drugs were found at either the same or at higher concentrations in the alkaline saliva than in the ultrafiltrate of plasma, a finding consistent with the theory that only the un-ionized form diffuses across the glandular epithelium. Accordingly, the salivary

Table 5

The relation between lipid solubility of the un-ionized compounds and the ratios found percentages of calculated ratios

	Un-ionized in plasma pH 7.4 %	Lipid solubility	Lipid solubility × % un-ionized	Experimental ratios as percentages of calculated values
Sulphanilamide	100	0.03	3	100
Sulphacetamide	5	0.1	0.1	1.2
Sulphadiazine	11	0.8	9	24
Barbitone	72	0.7	50	73
Sulphadimidine	50	3.2	160	58
Phenobarbitone	39	4.8	177	77
Pentobarbitone	80	28	2040	133
Reposal ®	61	~100	>6100	103

excretion of drugs in ruminants is governed by the same principle as in man however owing to the slightly acid or neutral nature of human parotid secretion (pH 5.5–7.8 SCHMIDT NIELSEN 1946), the concentration of acid drugs in human saliva is always lower than that in the ultrafiltrate of plasma (KILLMANN & THAYSEN 1955)

On comparing the theoretically computed and experimentally observed concentration ratios in saliva and ultrafiltrate of plasma (table 4) sulphanilamide and barbiturates show the closest agreement. As saliva does not stagnate in the parotid gland in the way that, for example, milk does in the udder but is excreted continuously attainment of equilibrium between drug concentrations in plasma and saliva will depend on the diffusibility of the compounds. As diffusibility for the drugs investigated depends not only on their degrees of dissociation but also on their lipid solubilities, both of these factors and their products are recorded in table 5 The experimental ratios, expressed as percentages of the theoretical values at equilibrium, are listed in the last column. As sulphanilamide is present entirely in the un ionized form in both saliva and plasma, this compound, in spite of its low lipid solubility reaches the theoretically computed ratio Of sulphacetamide only 1 % is un-ionized in plasma, and this compound has a low lipid solubility The diffusibility of this compound is therefore also low which may explain why the experimentally found ratio is only 1–2 % of the theoretical value In plasma the un-ionized fraction of the remaining compounds is between 11 and 80 % and their lipid solubility varies considerably The results in table 5 show that both dissociation and lipid solubility strongly affect the possibility of attaining diffusion equilibrium The demonstrated influence of lipid solubility is

consistent with the results from salivary excretion studies on non-electrolytes (AMBERSON & HÖBER 1932 BURGEN 1956)

In our study variations of secretion rate from 0 04 to 2–3 ml/min. did not reduce the concentration ratio S/P Ultr which is in agreement with the findings of KILLMANN & THAYSEN (1955) at secretion rates by man of 0 25 to 2.1 ml/min At secretion rates from 5 to 7 ml/min the ratio tended to fall for sulphadiazine and pentobarbitone. This tendency was, however less pronounced than the secretion rate dependence found by BURGEN (1956) who showed that the excretion of non-electrolytes in dog parotid saliva was passive and that the concentrations of some compounds fell with rising secretion rate, but that for other compounds it was initially lowered and later rose with rising secretion rate

It should be noted that the method used for determining barbiturates will also include any metabolites containing the barbituric acid ring Even in these acute experiments such metabolites might be formed and affect the results

Summary

In experiments on cows and goats it is shown that the concentration of sulphanilamide sulphadimidine, sulphaethoxypyridazine, sulphadiazine, pentobarbitone (mebumal NFN) barbitone (diemal NFN) Reposal ® and phenobarbitone (phenemal NFN) in the alkaline saliva was higher than or equal to the concentration in the ultrafiltrate of plasma The concentration in saliva depended on the pK_a value and the lipid solubility of the drug. The concentration ratio of saliva to ultrafiltrate of plasma was independent of variations in secretion rate from 0 04 to 2–3 ml/min. Sulphacetamide, which is mainly ionized (99 %) in bloodplasma and has a low lipid solubility was excreted in the saliva at concentrations below those in plasma.

A comparison of theoretically computed and experimentally observed ratios showed that the non protein bound and the un ionized form of sulphonamides and barbiturates tend to establish a diffusion equilibrium across the epithelium of the salivary gland In spite of the continous secretion and outflow of saliva, this diffusion equilibrium is reached by drugs with high lipid solubilities and low degrees of dissociation in blood.

Acknowledgement

The present study was supported by a grant from Godsejer Viktor A Goldschmidts Legat.

Sulphaethoxypyridazine was generously supplied by Lindinger Agro Co A/S Copenhagen, from American Cyanamid Company New York

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filtered. The residue is washed with ethanol/ether mixture. The ethanol/ether mixture is evaporated to dryness in an all-glass vacuum still. The residue is dissolved in 10 ml of carbon tetrachloride, and 0.5 g of solid cuprous iodide is added. The mixture is then shaken for 30 minutes in a mechanical shaker. The mixture is centrifuged, and the optical density of the clear yellow supernatant fluid is measured against carbon tetrachloride at 430 mμ.

Standards

12.5 25 50 and 100 micrograms of disulfiram in 10 ml volumes of carbon tetrachloride are treated with cuprous iodide, as described above.

Typical recoveries of TDD added to whole blood and plasma are shown in the table.

The procedure has been found to be readily applicable to faeces.

Table 1

The Recovery of Disulfiram added to Blood and Urine

	Quantity of Disulfiram added (micrograms)	Recovery %
Whole Blood (10 ml)	50	81
	100	81
	200	79
	500	78
Plasma (10 ml)	50	78
	100	74
	200	79
	500	81
Urine (500 ml)	250	71
	500	78
	1000	81

Urine

A 500 ml sample of urine is adjusted to pH 8 and extracted three times with 100 ml quantities of carbon tetrachloride. The carbon tetrachloride extracts are evaporated to dryness in an all-glass vacuum still. The residue is dissolved in 10 ml of carbon tetrachloride, and TDD is determined with cuprous iodide, as described above.

Typical recoveries are shown in the table.

Values exceeding the highest standard used may be encountered. Under such conditions, measured portion of the final carbon tetrachloride solution (after dilution to 10 ml with more carbon tetrachloride) should be used.

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From the Biochemical Laboratory Northern General Hospital,
Edinburgh Scotland.

The Determination of Disulfiram (Antabuse ® Tetraethyl Thiuramdisulphide) in Blood and Urine

By

S L. Tompsett

(Received August 31 1963)

Disulfiram (antabuse ® aversan ® cronetal ® a o) is used in the treatment of chronic alcoholism. After administration it may be found in body fluids unchanged, tetraethyl thiuramdisulphide (TTD) or in a reduced form diethyldithiocarbamic acid (DDC) (ELDJARN 1950 LINDERHOLM & BERG 1951) The literature describing its determination is not extensive. Below is reported a modification of the method of DOMAR *et al* (1949) This has particular advantages especially in the extraction procedure.

Procedure

Reagents

1 Ethanol/ether (1/1)
2. Standard solution of disulfiram in carbon tetrachloride (1 mg/ml)
3 Standard solution of sodium diethyldithiocarbamate in water (1 mg/ml) – freshly prepared.
4 Solid cuprous iodide.
5 Carbon tetrachloride.
6 10 % (w/v) sodium citrate in water
7 10 % ammonia.
8 5% (w/v) cupric sulphate in water

Method (TTD)

Blood

10 ml of whole blood or plasma are added to 100 ml of ethanol/ether mixture After standing at room temperature overnight the mixture is

Acta pharmacol et toxicol 1964 21, 23–35

From the Institute of Hygiene (Professor B. Lambert, M.D.), University of Aarhus, and from the Department of Biochemistry (Professor S. Darling, Ph.D.), the Royal Dental College, Aarhus, Denmark.

Paper Chromatography of Dicoumarol and some Related Substances. With a Method for the Quantitative Determination of Dicoumarol on Paper Chromatograms

By

Flemming Christensen

(Received January 17 1964)

Methods for identifying and determining dicoumarol (3,3'-methylene-bis-(4-hydroxycoumarin)) may be useful in experimental pharmacology and biochemistry as well as in forensic and clinical medicine. It should, however, be added that determinations of dicoumarol as a method of controlling anti-coagulant drug therapy has not been generally adopted, since blood coagulation studies have then been found more valuable.

Several methods for determining dicoumarol in biological materials have previously been described. Among these may be mentioned *colorimetric* (Pulver & von Kaulla 1948, Roseman & Green 1951, Lubran 1951) and *ultraviolet spectrophotometric* methods (Axelrod *et al* 1949, Weiner *et al* 1950). In a few investigations of the fate of dicoumarol in different animal species *tracer methods* with dicoumarol labelled by ^{14}C have been used, (Lee *et al* 1950, Hausner *et al* 1951). All of these methods have in common that they are not entirely specific for dicoumarol, in particular that they will not permit distinction between dicoumarol and its chemically related metabolites. Recently a method for determining dicoumarol in the plasma by *infrared spectrophotometry* (Stubbs *et al* 1962) has been reported. The method seems to be more specific for dicoumarol than any of the others mentioned. It has not been shown whether or not this method can distinguish between dicoumarol and its metabolites. For a final identification of dicoumarol *crystallographic* values have been reported by Rose & Williams (1961).

In connexion with an investigation into the fate of dicoumarol in the rat it was found desirable to determine dicoumarol specifically even in the presence of its metabolites. It was also found desirable to be able to

Method (DDC)

Urine

To 10 ml of urine are added 1 ml of 10% sodium citrate, 1 ml of 10% ammonia, 1 ml of 5% copper sulphate and 10 ml of carbon tetrachloride. The mixture is shaken vigorously for 10 minutes and then centrifuged. The carbon tetrachloride layer is separated, and the optical density is read against an appropriate blank at 430 mμ.

Blood

To 2 ml of serum are added 8 ml of water and 1 g of pancreatin (British Drug Douses Ltd) and the mixture is incubated at 37°C for 12 hours. The determination is then carried out as described for urine.

Standards

25 50 100 and 200 micrograms of sodium diethyldithiocarbamate in 10 ml quantities of water are treated in exactly the same manner as described above.

Table 2

The Recovery of DDC added to Urine and Plasma

	Quantity of DDC added (micrograms)	Recovery %
Urine (10 ml)	50	74
	100	81
	200	83
Plasma (2 ml)	25	74
	50	76
	100	78

Summary

Disulfiram is extracted from plasma whole blood or fæces with ethanol/ether Disulfiram or diethyldithiocarbamic acid is determined photometrically at 430 mμ in the evaporated extract after addition of cuprous iodide.

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technique with system 1 for the first direction and system 3 for the second direction has been found helpful in separating dicoumarol and some of its metabolites obtained from biological materials.

Procedures for detecting compounds on the chromatograms

In the detection of the compounds listed above on paper chromatograms the colour reactions found to be useful were those with

1 *Diazotized o-dianisidine*

This colour reaction was found to be of value for differentiating various compounds of the coumarin type. Preliminary studies also indicated it to be valuable for demonstrating the presence of the metabolites of dicoumarol isolated from rat tissues on the paper chromatograms.

The reaction was carried out as described below A solution of o-dianisidine was prepared by dissolving 2.0 g of o-dianisidine (BDH analytical grade) in 1 litre of 1 M-HCl. After standing overnight the solution was filtered and was found to be usable for weeks when kept at room temperature. The spray reagent was prepared by mixing 25 ml of this solution with 1.5 ml of a 5 % (w/v) aqueous solution of sodium nitrite, keeping this mixture for about 5 min. at room temperature before adding 25 ml of a 2 M solution of K_2HPO_4. The pH of the resulting mixture should be between 6 and 7 The chromatogram was sprayed evenly with the reagent. To keep the background bright, this treatment may be followed in 15-30 min. by a second spray with 3 M-HCl. It should be noted that many spots will then change colour

2. *Diazotized sulphanilic acid*

Coupling phenolic compounds with diazotized sulphanilic acid is as a rule done at an alkaline reaction, but some compounds may couple at even a slightly acid reaction. Dicoumarol is among them and couples best when the reaction is at about pH 6. Most of the other substances studied did not couple at all at an acid reaction. When demonstrating such compounds on paper chromatograms, coupling should therefore be done at an alkaline reaction, since few phenolic substances then fail to react. However when determination of dicoumarol is required coupling should be done at a slightly acid pH. For quantitative dicoumarol determination this procedure was adopted. It is described in the section on quantitative determination of dicoumarol.

For coupling at an alkaline reaction the procedure was to mix 25 ml of a 0.45% (w/v) solution of sulphanilic acid in 1.0 M HCl and 1.5 ml of a 5 / (w/v) aqueous sodium nitrite solution and allow the mixture to stand at room temperature for about 5 min. Then 25 ml of a 2 M aqueous solution of Na_2CO_3 were added, and the mixture was shaken. The chromatogram was then immediately sprayed with the reagent. This treatment may be followed in 15–30 min. by a second spray with 3 M HCl to keep the background bright, but it should be noted that many spots will then change colour and some may even disappear entirely on this treatment.

Apart from the colour reactions just mentioned, additional help in the detection of the spots on the chromatograms may be obtained by exposing the chromatograms to the light of an ultraviolet lamp. In our study a Philips ultraviolet lamp with a

demonstrate the presence of and to separate such substances. The work of HAIS (1951) indicated paper chromatography as a means of solving the problem As preliminary studies seemed promising, further investigations along the same lines were undertaken The possibility of separating and even identifying different anti-coagulant drugs of the 4-hydroxycoumarin type was also considered.

Based upon paper chromatography and colour reactions, methods for separating and detecting some 4-hydroxycoumarin derivatives as well as some chemically related compounds have been worked out. Also a method is given for quantitative determination of dicoumarol on paper chromatograms in amounts ranging from 2 to 30 µg. The application of the last mentioned method to biological materials is the subject of another paper being prepared for publication

Experimental Methods

Compounds studied

The compounds used in this study were

Dicoumarol (NFN) (3,3 -methylene bis-(4-hydroxycoumarin))

Aethyli bicumacetas (NFN) = tromexan ® (3,3 -carboxyethylmethylene bis (4-hydroxycoumarin))

Phenprocumonum (NFN) = marcoumar ® (3-(1 phenylpropyl)-4-hydroxycoumarin)

Aceno cumarolum (NFN) = sintrom ® (3-(α p-nitrophenyl-ß-acetyl-ethyl)-4-hydroxycoumarin)

Phenindion (NFN) (2-phenyl indandione 1,3)

4-Hydroxycoumarin (Fluka purum)

4 7 Dihydroxycoumarin was prepared according to the method of SONN (1917) as modified by HEILBRON & HILL (1927)

Salicylic acid (ANALAR, analytical grade)

o-Coumaric acid (BDH, analytical grade)

Paper chromatographic procedure

The paper chromatographic procedure was identical in the experiments carried out to separate the compounds listed above and in the experiments on quantitative determination of dicoumarol on chromatograms.

Whatman paper No 1 without any prior treatment was used throughout the study

The papers were developed at room temperature by the descending technique. The compounds were applied to the papers dissolved in either absolute alcohol or aqueous 0 1 M NaOH As a rule amounts of 5–30 µg of the individual compounds were applied to the paper in spots not exceeding 5–7 mm in diameters.

The solvent systems used for developing the chromatograms were

System 1 n-butanol 3 M aqueous ammonia 1 1 (v/v)

System 2 Ethyl acetate 3 M aqueous ammonia, 1 1 (v/v)

System 3 Benzene acetic acid water 2 2 1 (v/v)

One-dimensional chromatography was used in this study but the two-dimensional

on the other hand the chromatogram must not be overloaded with fluid, as the coloured material of the spots is soluble in the water phase.

Drying the chromatograms

The chromatogram is placed under a hood for drying. Depending on the conditions, this will take 15 to 30 min. It is not necessary for the chromatogram to be absolutely dry before further treatment. However it should be possible to mark the coloured spots on the chromatograms with a soft pencil. This should be done in such a manner that the individual spots are encircled, leaving a zone of at least a few millimeters free of coloured matter between the spot and the pencil marking. As a rule 3 areas of the same dimensions as the areas containing dicoumarol are marked on the chromatogram to serve as blank spots. It is recommended if possible for all the paper pieces cut from a chromatogram to be of the same dimensions.

To select the spots that contain dicoumarol one can be guided by the colour as well as by the position of the spots relative to spots of dicoumarol standards on the same chromatogram.

Elution of coloured matter from the paper

The areas already marked on the chromatogram are next cut from the paper and the paper pieces are placed in test tubes containing 5 ml of 0.1 M HCl. A rubber stopper is placed in each of the tubes, which are carefully inverted 10 times. They are then left to stand, the invertion being repeated every 15 min. In one hour the elution of the coloured matter from the paper pieces is complete, and the absorbances of the individual solutions may be determined.

Spectrophotometric determination

The absorbances of the solutions are determined in a spectrophotometer. In our study a Zeiss spectrophotometer PMQ II and glass cells with a light path of 1.00 cm were used throughout. The absorbances were measured at 415 mμ with 0.1 M HCl in the reference cell.

Calculation of the dicoumarol content in unknown samples

The absorbances of the unknown as well as of standard dicoumarol samples are first corrected for the blank value. Because of the proportionality between the dicoumarol content of the samples and the corresponding absorbances corrected for the blank value, it is an easy matter to calculate the dicoumarol content of unknown samples.

maximum energy emission at about 360 mμ was used. Some of the compounds studied under these conditions showed a detectable fluorescence, and one compound showed quenching of the background fluorescence

Quantitative determination of dicoumarol on paper chromatograms

The principles for determining the amount of dicoumarol in the spots on paper chromatograms are described below After development by the one-dimensional technique the paper chromatograms are dried and sprayed with diazotized sulphanilic acid The spots containing dicoumarol are identified by their R_f-values and their colours The coloured spots are cut from the chromatograms blank spots of the same dimensions as those containing dicoumarol are also cut from the chromatograms. The coloured material of each spot is eluted from the paper and the absorbance (extinction) of the resulting solution is determined spectrophotometrically at 415 mμ. The extinctions of unknown as well as of standard dicoumarol samples are corrected for the blank value It is then possible to calculate the amount of dicoumarol in the unknown sample

Details of the procedure are given below

The *chromatographic procedure* has already been mentioned Development of the chromatograms is performed as described with solvent system no 1 (butanol aqueous ammonia) Only one-dimensional technique should be used in the quantitative determinations of dicoumarol on chromatograms, *since it is necessary to use standards that have been* treated exactly in parallel with the unknown samples.

Colour reaction with diazotized sulphanilic acid

The solutions prepared are

Sulphanilic acid (0 45% w/v) in 1 0 M HCl is prepared by dissolving 4 5 g sulphanilic acid (Merck *pro analysi*) *in 1 0 M HCl and making the* solution to 1 litre by 1 0 M HCl

2 M-dipotassium hydrogen phosphate (Baker analyzed reagent) and 5% (w/v) aqueous sodium nitrite (Merck, *pro analysi*) are also prepared.

The spray reagent is made by cooling 25 ml of the sulphanilic acid solution on a water ice bath until the temperature is about 0 Then 1 5 ml of sodium nitrite solution are added the mixture is left on the bath for a further 10 min and 20 ml *of dipotassium hydrogen phosphate solution* likewise cooled to about 0 are added. The chromatogram is immediately sprayed with the reagent. In a preliminary test the of the spray solu*tion* should be checked as being between 6 0 and 6 2. When the chromatogram has been sprayed with the reagent it should be evenly wet

Table 1

Colour reactions, fluorescence and R_F-values of dicoumarol and related compounds.

Compound	Colour with diaz. sulphanilic acid pH approx. 9	Colour with diaz. o-dianisidine pH approx. 7	Colour of fluorescence excited by uv-light	Solvent systems*) Syst. 1	Syst. 2	Syst. 3
Dicoumarol	orange	purple	yellow-green (weak)	0.76	0.31	0.96
Tromexan ® Aethyl biscumacetate (NFN)	orange	red	yellow-green (weak)	0.75	0.23	0.96
Marcoumar ® Phenprocoumone (NFN)	orange	bright yellow	bright blue	0.89	0.25	0.93
Sintrom ® Acenocoumarole (NFN)	white	white	brownish	0.88	0.28	0.80
Fenindione *)	yellow	yellow-orange	Q *)	0.77	0.04	0.96
4-Hydroxycoumarin	yellow	purple	green (weak)	0.57	0	0.32
4,7 Dihydroxycoumarin	orange	dark purple	green (weak)	0.02	0	0.04
Salicylic acid	yellow-green	no colour	dark blue	0.60	0.05	0.82
o-Coumaric acid	dark orange	yellow-brown	bright blue	0.20	0	0.43

*) System 1: n-butanol 3 M aqueous ammonia 1:1 (v/v)
System 2 Ethyl acetate 3 M aqueous ammonia 1:1 (v/v)
System 3 Benzene acetic acid water 2:2:1 (v/v)

*) The solution of the compound in 0.1 N NaOH is deep red.

*) Q means quenching of the background fluorescence of the paper

Results

1 *Chromatographic behaviour and colour reactions of compounds studied*

The results of the chromatographic examination of the compounds mentioned are given in table I from them it will be evident that the various compounds may be distinguished from each other by the methods mentioned in the preceeding section R_f-values alone will not permit identification of a given compound with certainty but along with the o-dianisidine colour reaction which has proved especially valuable they permit identification with a high degree of probability The ultraviolet fluorescence exhibited by many of the compounds studied may also be useful for identification. The sulphanilic acid reaction may help to demonstrate the presence of compounds of the type studied on the chromatogram, but the colours obtained do not help much in identification However a positive colour reaction when coupling is carried out at an acid pH is fairly characteristic of dicoumarol and tromexan

2 *Quantitative determination of dicoumarol on paper chromatograms*

The method as described in the section on quantitative determination of dicoumarol was accepted only after the effects of several factors,

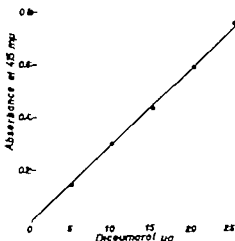


Fig. 1 Relation between amount of dicoumarol applied on a chromatogram, developed in system 1 (butanol – aqueous ammonia) and the absorbance at 415 mμ. The determinations were carried out as described in the text, and the individual absorbances were corrected fo the blank values. Each point on the curve represents the mean of two determinations.

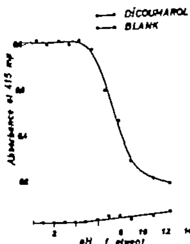


Fig. 2. Dependence of absorbance at 415 mμ on the pH of the elution solvent. The values of the absorbances are corrected for the blank values. Here 25 μg-samples of dicoumarol were applied to the chromatograms in spots of equal sizes, and the chromatograms were not developed before the colour development. The elution of the coloured material from the spots was carried out by means of different buffers at pH-values indicated on the curve. The procedure was otherwise as described in the text.

alkaline solution. The dependence of the absorbance at 415 mμ on the pH of the elution solvent is shown in fig. 2.

The absorption spectrum of the dyestuff in 0.1 M HCl solution, as well as the spectrum of a blank solution, are shown in fig. 3. The absorption maximum of the coupled product with dicoumarol, as already mentioned, is at 415 mμ.

The intensity of the colour development as measured by the absorbance was found to depend on the concentration of the sulphanilic acid used. This is illustrated in fig. 4. It should be noted that the concentrations given in the figure are the concentrations of the sulphanilic acid solution used for mixing with the sodium nitrite and potassium hydrogen phosphate.

The most important effect on the intensity of the colour development was that of the pH of the final spray reagent. This dependence is illustrated in fig. 5. It is seen that the intensity is at a maximum when the pH is between 6 and 7 and that a rapid decline occurs outside this range.

Several other factors were also examined. Wide variations in the amount of nitrite used did not affect the results significantly. Washing the papers before chromatography did not seem to be of any benefit in reducing variations in the absorbances of the blank tests. It is, however, recommended that any contamination of the papers with phenolic substances should

Table 2

Reproducibility and accuracy of the paper chromatographic determination of dicoumarol. The results given are from identical experiments carried out on 3 consecutive days. In each experiment 2 sheets of Whatman No 1 paper (50 × 40 cm) were used. On one of these 8 samples containing 5 μg of dicoumarol were spotted, on the other similarly 8 samples containing 25 μg. The chromatograms were treated in parallel. They were developed in n-butanol–3 *M* NH_4OH = 1:1 (v/v) and the quantitative determinations were carried out as described in the text. The absorbances corrected for their blank values were calculated per 1 μg of dicoumarol. The mean and the standard deviation for each group are given.

Day of determination	Absorbances (× 10^3) calculated per 1 μg of dicoumarol (Mean ± standard deviation)	
	5 μg dicoumarol	25 μg dicoumarol
1	29.00 ± 0.87 (8)	28.97 ± 0.18 (8)
2	28.20 ± 0.28 (8)	29.32 ± 0.16 (8)
3	28.60 ± 0.85 (8)	27.96 ± 0.22 (8)

especially on the intensity of colour development, had been throughly investigated. The results of these experiments are given below as well as results *showing the accuracy of determining dicoumarol in samples of* known content.

With the method adopted, proportionality between dicoumarol content of samples applied to the chromatogram and absorbance, was obtained (fig. 1). This proportionality was demonstrated with amounts of dicoumarol from 5 μg to at least 30 μg.

The accuracy as well as the reproducibility of dicoumarol determinations by the method described are illustrated in table 2. From this table it may be seen that the day-to-day variations are relatively small and insignificant. Further, it may be seen that the accuracy of dicoumarol determinations on a given chromatogram is such that the standard deviation does not exceed 1% for dicoumarol in amounts of 25 μg and 3% for amounts of 5 μg. Quantities as small as 2 μg may be determined with an accuracy of about 10–15%.

As already indicated, several factors *were shown to have a profound* effect on the intensity of the colour development as well as on the accuracy of the determination of dicoumarol. Among these some of the most important are mentioned below.

As the coupled product of dicoumarol and diazotized sulphanilic acid is an azo-dyestuff, pH-dependence of the colour shade was expected and the dyestuff did exhibit such dependence, being bright yellow in acid solution and orange in alkaline solution. The absorbance of the bright yellow solution was found to be at a maximum at 415 mμ and was found to be *much* higher than that of an equimolar solution of the dyestuff in

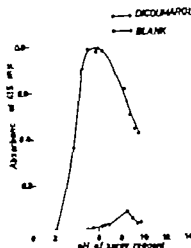


Fig. 5 Dependence of the absorbance at 415 mμ on pH of the spray reagent. The values of the absorbances are corrected for the blank values, which are also given. The pH values indicated are obtained by using different buffers instead of the phosphate buffer usually employed Otherwise the procedure was as described in the text. Samples containing 25 μg of dicoumarol were applied to the chromatograms in spots of equal dimensions. Development of the chromatograms was not performed.

be avoided, as this may totally invalidate the determinations. In controlling the variations in the values of the blank tests it should be noted that an increase in these values with time from spraying the chromatogram and beginning the elution always occur On the other hand, a slight increase in the intensity of the colour of dicoumarol spots is noted during the first 10 to 15 minutes after the chromatogram has been sprayed. It should be added that it is of prime importance for the standard dicoumarol spots to be treated exactly in parallel with the unknown samples. This means that the samples should also be chromatographed, not applied to the chromatogram after development has been performed, because the intensity of the colour development is less for spots that have been chromatographed than for those that have not.

Discussion

As already mentioned, principles for separating and demonstrating 4-hydroxycoumarin derivatives by paper chromatography were introduced by HAIS (1951). He even described methods for determining such compounds in blood and urine by using this principle along with the

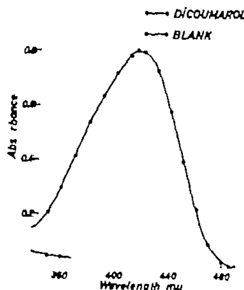


Fig. 3 Absorption spectrum for the coupled product of dicoumarol with d azotized sulphanilic acid. A 25 μg sample of dicoumarol was applied to a chromatogram The colour reaction and elution were performed as described in the text. The absorbances of the resulting solution were determined at the wavelengths indicated and corrected for the blank values, which are also given.

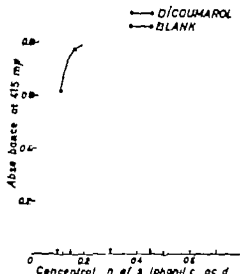


Fig. 4 Dependence of the absorbance at 415 mμ n concentration of the lphan lic acid used The concentrations given are those f the s lphanilic acid solut ns used for m xing with solutions of sodium nitrite and dipota sium hyd ogen phosphate. The absorbances indicated are corrected for thei blank values, which are al o given Development of the chromatograms was not performed in this experiment Each sample contained 25 μg of dicoumarol and all spots were f equal d mensions. The proced re wa otherwise a described n the text.

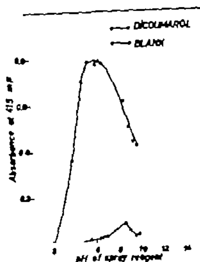


Fig 5 Dependence of the absorbance at 415 mμ on pH of the spray reagent. The values of the absorbances are corrected for the blank values, which are also given. The pH-values indicated were obtained by using different buffers instead of the phosphate buffer usually employed. Otherwise the procedure was as described in the text. Samples containing 25 μg of dicoumarol were applied to the chromatograms in spots of equal dimensions. Development of the chromatograms was not performed.

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Discussion

As already mentioned, principles for separating and demonstrating 4-hydroxycoumarin derivatives by paper chromatography were introduced by Hais (1951). He even described methods for determining such compounds in blood and urine by using this principle along with the

colour reaction with diazotized 4-nitraniline However this method makes use of a visual comparison of spots with known and unknown amounts of the compound to be studied, so that its accuracy is limited. HAIS *et al* in a later communication (1958) have further contributed to our knowledge of the chromatographic behaviour of some 4-hydroxycoumarin derivatives

In our investigation the use of paper chromatography and of colour reactions with diazotized aromatic amines has been applied to the problems of separating and demonstrating some 4-hydroxycoumarin derivatives as well as to determining dicoumarol The methods, as presented here are not directly applicable to biological materials but this problem is to be treated in another paper It seems however clear that the paper chromatographic methods described here may be valuable in studying the metabolic fate of coumarin anti-coagulant drugs in man and animals and may also be useful in forensic medicine

The results indicate that identification of the anti-coagulant drugs studied may be possible with a fairly high degree of probability even when the amounts available are to be reckonned only in micrograms. The use of the colour reaction with diazotized o-dianisidine is particularly helpful in this situation By the use of the sulphanilic acid colour reaction it has further been possible to determine minute quantities (*e g* 2–30 µg) of dicoumarol on chromatograms with a high degree of accuracy As to the specificity of determining dicoumarol by means of paper chromatographic methods described, no final statement can at present be made, but it seems reasonable to assume that this may be fairly high and in particular may distinguish between dicoumarol and its as yet unknown metabolic products.

Summary

Methods based on paper chromatography and colour reactions with diazotized aromatic amines have been worked out for separating and detecting some 4-hydroxycoumarin anti-coagulant drugs as well as some chemically related compounds The methods described may permit a preliminary identification of the substances examined and may be helpful for studying the fate of dicoumarol in the organism.

Further a method for the quantitative determination of dicoumarol on paper chromatograms is described The method can determine amounts of 2–30 µg of this substance with a reasonable degree of accuracy The application of this method to biological materials is to be described in a succeeding paper

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Behavioural Response of Rats During Inhalation of Trichloroethylene and Carbon Disulphide Vapours[1,2])

By

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(Received January 20, 1964)

Previous studies from this laboratory have shown that repeated inhalation of vapours of certain industrial solvents will produce an inhibition of the conditioned reflex in rats trained for a pole-climb response (GOLDBERG *et al* 1962, GOLDBERG *et al* 1964) Because of their commercial importance, trichloroethylene (TCE) and carbon disulphide (CS_2) have been selected for further behavioural investigations.

According to the extensive investigations of Grandjean and his coworkers (ROSSI & GRANDJEAN 1959 GRANDJEAN 1960 ZAHNER, BATTIG & GRANDJEAN 1961) repeated inhalation of TCE vapour at concentrations between 200 and 800 p p m. modified the psychological equilibrium in rats as manifested by an increase in excitability or disinhibition Animals showed a significant increase in spontaneous response before a conditioned stimulus in a food motivated programme In rats trained for an avoidance situation, repeated inhalation prolonged response latency but did not inhibit avoidance response In a spontaneous alternating situation repeated inhalation of 400 to 800 p p m of TCE reduced the frequency of alternation and increased running speed in a T maze higher concentrations markedly depressed running speed BATTIG & GRANDJEAN (1963) summarized their behavioural studies by indicating that repeated inhalation of TCE vapour at concentrations between 200 and 800 p p m improved or impaired performance, depending on the test applied, whereas concentrations above 800 p p m usually resulted in impairment. KHORVAT & FORMANEK (1959) reported that rats exposed

1) This work was supported in part by a grant from the National Institutes of Health (OH 16)

2) A portion of this report was presented at the Society of Toxicology Meeting at Cincinnati, Ohio, May 1963

to 75 p.p.m. of TCE manifested a reduction in the time of latency of a food-motivated reaction accompanied by an increase in motor excitation and discriminatory impairment. KHORVAT & FORMANEK (1954) have also described the behavioural response to repeated vapour inhalation of CS_2 after a concentration of about 1000 p p m A prolongation in response latency in a food reward situation was observed.

We wished to study the behavioural effects of TCE and CS at con centrations nearer to their threshold values (T L.V), which are 100 and 20 p.p.m. respectively (American Conference of Governmental Industrial Hygienists 1963). We desired also to have the animals inhale the vapours while performing their behavioural tasks without disturbance from handling. For simplicity of programming, recording and interpreting, a discrete or noncontinuous avoidance situation was utilized. This technique permits behavioural study over longer periods of time than previously utilized, while automatically recording the performance of the animal. We consider this experimental design is somewhat analagous to that of a factory employee working in a low vapour background while performing his occupational tasks.

Methods

Animals. Male Carworth Farms Elias (CFE) rats were reared in our colony and allowed food and water *ad libitum* except during training and experimentation. At the time of use, the animals weighed between 350 and 450 g and were about 125 to 150 days of age.

Behavioural techniques Discrete avoidance studies were performed in a 10-compartment rat-shock box (Lehigh Valley Electronics) with white noise background. Details of this procedure have been presented elsewhere (GOLDBERG *et al.* 1963). Ten rats were trained to 95 or higher efficiency in avoiding shock, in sessions of 120 avoidance trials/hour in accordance with the schedule 18-second intertrial, 10-second cue light, 2-second shock (3mA). Results are given both as the number of shocks received during an experimental period, expressed as group average, and as the number of rats in the group that show a significant reduction in avoidance efficiency ($P < 0.01$ from normal grouped or individual baseline performance). Once trained, the animals retained a stable baseline for many months under the conditions described below

Inhalation chamber and vapour delivery A 566-litre inhalation chamber was constructed of hard-board (Fig. 1). Vapours were generated by a motor-driven syringe, which delivered a constant flow of the test liquid down an electrically heated spirally corrugated Pyrex tube connected to the air inlet of the chamber (CARPENTER *et al.* 1949). The shock-box, placed within the chamber was designed to facilitate the free passage of air or vapour in air (Fig. 2). With the door in position, the chamber was exhausted under slight negative pressure at a rate of approximately 260 l/min. The animals were subjected to identical chamber conditions during control periods except that no vapour was generated.

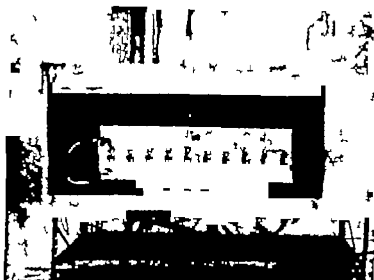


Fig. 1 10 Compartment rat-shock box in inhalation chamber with door removed.

Vapour analysis Relative concentrations of TCE vapour in each of the compartments and at various locations outside the shock box were determined with a Zeiss portable interferometer Maximal difference in concentration between any 2 points was about 10 %. With calibrated gas chromatograph (F and M Model 609) a similar distribution study was performed. A mean concentration of 128 ± 11 (S D) p p.m.

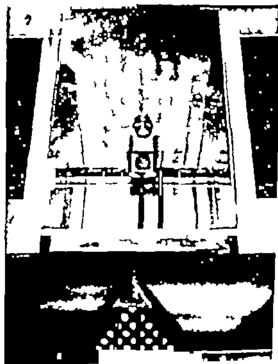


Fig. 2. Internal view of an individual compartment in the rat-shock box.

of TCE was obtained. For convenience this concentration will be designated as 125 p.p.m. The mean concentration of CS_2 vapour as determined by the calibrated interferometer was 194 ± 5 (S.D.) p.p.m. This concentration will be referred to as 195 p.p.m.

Procedure. Each vapour study was composed of 3 phases pre-exposure control, vapour exposure and post-exposure control (recovery phase). In the pre-exposure control phase, selected rats were acclimatized to perform their behavioural tasks when the shock box was placed within the inhalation chamber Animals were allowed to remain in the chamber for 4 hours a day during each of the 3 phases of the study During the first 2-3/4 hours, a house light in the chamber was on, and no trials occurred. There was then a 15-minute avoidance warm-up period, in which the house light was off and a white noise background turned on. Such a regimen continued for the remaining hour during which the number of shocks taken was recorded. The animals inhaled vapour for 4 hours a day 5 days a week for several weeks at the same time each day

Results

Trained rats were easily acclimatized to work during the fourth hour in their various environmental compartments. After a few days of this schedule, with a suitable warm-up period before recording, the avoidance efficiency of the animals was identical with that obtained before acclimatization.

Trichloroethylene During an 8-day pre-exposure control period, the 10 rats revealed an average of 4 4 ± 1 6 (S D) shocks/hour during the

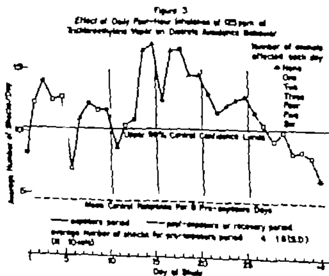


Fig. 1. Effect of daily four hour inhalation of trichloroethylene vapour (125 p.p.m.) on discrete avoidance behaviour of rats.

fourth hour in the chamber Upper 99 / control confidence limits for this period were obtained by adding the shock average to the product of the standard deviation and a t value of 3 499 This will yield a shock average of $P < 0\ 01$ when compared to the pre-exposure control period average for the group Any value above this limit obtained during the exposure period is significantly higher than the control response Results of repeated inhalation of 125 p p.m are given in Fig. 3

The mean pre-exposure average and the statistical cut-off point are shown as horizontal lines. The vertical bars separate the 5-day periods of inhalation and signify a week-end rest for the animals Individual points for each day of the study after the pre-exposure phase are characterized by symbols and signify the number of rats that showed a significant ($P < 0\ 01$) difference from their individual control performance The level of this significance is obtained in a way similar to that described above for the entire group This method of presenting results represents both a quantitative and qualitative expression. During a study of 25 exposure days, a significant increase in the number of shocks taken by the group was observed on 22 days No significant effect was observed during the first day of each of the first 3 weeks of study

The number of animals affected on any given day varied from 1 to 6 in the group Several variations in response patterns were obtained during the inhalation phase A few animals were continuously affected throughout the entire study some were affected early but developed rapid tolerance some were not affected until the third week of study and two never manifested an alteration in avoidance efficiency Thus, it should be pointed out that the 6 animals affected on dav 18 of study do not necessarily include all rats affected on day 3 of study The original behavioural pattern of an individual rat bears no relationship to the degree of sensitivity of altered behaviour induced by TCE vapour Although the results are not presented here it was apparent in several of the animals that the number of lever presses during the intertrial period was increased during the exposure phase These are the responses not associated with avoiding shock and may be attributable to an impairment in discrimination or an increase in anxiety in the rats

A gradual return to normal avoidance responding was observed during the recovery phase Behavioural effects did persist for several days, but were reversible All animals maintained a mature body weight throughout the entire study

Carbon Disulphide Results in our laboratory (unpublished) revealed that repeated inhalation of 100 p p m of CS_2 vapour was without effect on discrete avoidance behaviour and body weight Thus we selected 200 p p m., which is 10 times the T L.V for CS_2 for these investigations.

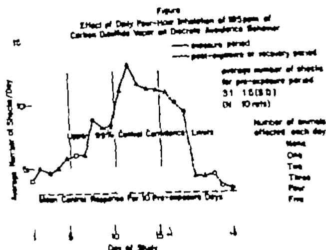


Fig. 4. Effect of daily four hour inhalation of carbon disulphide vapour (195 p.p.m.) on discrete avoidance behaviour of rats.

Animals in this study showed an average of 3.1 ± 1.5 (S.D.) shocks/hour for a 10-day pre-exposure control period. Upper 99 / control confidence limits were obtained for grouped and individual rats as described above. The results of repeated inhalation of CS_2 are given in Fig. 4.

Significant increase in the number of shocks received in grouped animals was observed only during the last 8 days of a 15-day exposure period. The number of animals affected progressed from 1 to 5 throughout the course of repeated inhalation. With few exceptions, the same animals affected in the early days of the study continued to show behavioural alteration during the remainder of the study. Concomitant with avoidance disruption, there was an average weight loss of 29 grams in these mature rats at the end of the 3-week exposure period. This accounted for a 6.5 / reduction in initial body weight ($P < 0.01$). Inhalation of vapour was terminated at this time. An average weight loss of 3.5 / was in fact obtained after 4 exposures to CS_2 vapour at this concentration. As may be seen from the figure, evidence of "behavioural toxicity" had disappeared by the end of the recovery period. At this time, the animals had regained only half of their weight loss.

Discussion

Behavioural techniques, largely characterized by a Pavlovian approach, have been utilized for several years by many European investigators for

safety evaluation of industrial chemicals. Review of translations of Soviet literature (LEVINE 1960) on air pollution revealed much of the methodology A recent publication expounded the many methods currently available as guides to the setting of threshold limit values (STOKINGER 1963). It is not of interest at this time to pursue further this aspect, except to note that behavioural techniques are presumably widely used in the Soviet Union for setting the maximal allowable concentrations of vapours in their industries The philosophy of the Russian toxicologists was interpreted by RUFFIN (1963) who mentioned that the conditioned response may be the most sensitive method for demonstration of harmful effects of industrial chemicals upon animals not because the nervous system is necessarily more susceptible than other organ systems, but because maintenance of the conditioned reflex is considered as being dependent upon the total internal state of the organism RUFFIN holds that Soviet workers may extend this theory to a conclusion that subtle change in any organ function may result in alteration in the pattern of an established conditioned reflex.

It is entirely possible that a more sophisticated methodological approach to the study of behavioural response may reveal such subtle changes With the methods we have employed we find no consistency in claiming such an interpretation We have demonstrated an effect with TCE vapoor near its 1963 T L.V so have several other investigators. However in the study of CS_2 we do not observe this a concentration 5-times the T L.V (100 p p m) was without effect on the type of behavioural studied At 195 p p m. effects on behaviour and body weight were obtained Thus, from our behavioural and inhalation schedules, evidence of toxicity revealed by behavioural technique was not necessarily more sensitive than another measure of injury

The fact that TCE and CS_2 impaired trained animal performance in repeated inhalation is not startling, in view of many published reports. The relevant features of the experimental design employed namely low concentrations study during exposure and use of a manipulative task, provide an analogy to possible human experience One salient difference in our approach from those described in earlier reports is that during the behavioural testing phase the animals are not handled by the experimenters. Failure of earlier reports to demonstrate impairment in behavioural performance after low level inhalation of these solvents could be attributable to an effect on the animals from handling, which could compensate for chemical effectiveness. It has been demonstrated that stressed animals elaborate an increase in adrenal cortical steroid output which is higher than in unhandled animals (BARNETT 1963) Thus animals that are not handled may be more sensitive to drug effects. The inter

relationship of chemical stress, adaptative patterns and behavioural responses obviously requires more extensive investigation. The behavioural effects observed in these selected animals may not be identical to those obtained in a random animal population. It is conceivable that the degree of stress imposed upon the animals during the training phase of the behavioural study may dictate the relative susceptibility of these rats during chemical "stress".

Many of the reported investigations on the behavioural effects of vapours of industrial solvents fail to include a post-exposure or recovery phase. In animals maintained by positive reinforcement, it is difficult to justify exclusion of this phase. Reversibility of chemical effect and animal deterioration or extinction have not been fully explored in such investigations. Although many methods are currently available to an investigator for studying toxicity by the behavioural approach, a more thorough evaluation of such methods may be necessary.

Summary

Vapours of trichloroethylene or carbon disulphide have been inhaled by rats selected on the basis of successful training to be 95% or more efficient in preventing shock on a discrete avoidance behaviour schedule. After suitable acclimatization, the animals performed 5 days a week during the last hour of a 4-hour inhalation period. Animals were exposed to 125 p.p.m. of trichloroethylene for 5 weeks and to 195 p.p.m. of carbon disulphide for 3 weeks. Both agents caused significant alterations in behaviour; however carbon disulphide at this level also produced a significant loss in body weight. During the first few weeks of trichloroethylene inhalation, a reduced effectiveness was observed on Mondays, possibly attributable to elimination of the chemical during a week-end rest. Innovations in experimental design permitted behavioural evaluation during inhalation without the handling of animals and also inclusion of a post-exposure recovery period. Behavioural effects with either vapour were completely reversible during the recovery phase.

Acknowledgments

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Studies on the Salivary Gland Hypertrophy Induced in Rats by Isoprenaline

By

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(Received January 27 1964)

SELYE, VEILLEUX & CANTIN (1961) observed that treatment of the rat with repeated sublethal doses of isoprenaline caused an excessive hypertrophy of the salivary glands. The reason for this enlargement was considered to be proliferation and hypertrophy of the parenchymal cells. This effect on the cell-size of the salivary glands of rats and mice is now well established (SELYE, CANTIN & VEILLEUX 1961 BROWN-GRANT 1961 SCHNEYER 1962). Accelerated morphological differentiation has also been shown in parotid and submaxillary glands of early postnatal rats (SCHNEYER & SHACKLEFORD 1963)

Our work was mainly undertaken to study the effect of various adrenolytics on salivary gland hypertrophy induced by isoprenaline. Much of this paper deals with the dose response relationship of this reaction

Methods

Male Sprague-Dawley rats of 120 to 130 g initial weight were kept at about 23°C temperature and fed on standard rat pellets and water *ad libitum*. Isoprenaline sulphate (Lääke Oy Turku) was injected daily subcutaneously at about 9 a.m. and in some experiments again about 9 hrs. later As additional treatment dichlorisopropylnoradrenaline hydrochloride (Aldrich Chemical Co Inc. Milwaukee, Wisc.) or phenoxybenzamine hydrochloride (Smith, Kline & French Labs. Philadelphia, Pa. benzylyl chloridum NFN) was given subcutaneously 30 min. before the isoprenaline and another dose of dichlorisopropylnoradrenaline 9 hrs. later All doses are given in terms of their relevant free bases.

About 24 hrs. after the last injection of isoprenaline, the rats were anaesthetized with ether and bled by cutting the abdominal aorta the submaxillary parotid and major sublingual glands were then removed. Their total fresh weights are given in the text below Specimens of the salivary glands were frozen for staining with haematoxylin-eosin, but no attempts were made to examine the histological changes occurring under the various experimental conditions. Results were analysed for significance by Student's t-test.

Results

A *Effect of isoprenaline alone*

Both the submaxillary and the parotid glands seemed to take part in the hypertrophy On the other hand, the weight increase in the sublingual gland was not clear-cut The size of the extraorbital lachrymal glandwas unchanged There was a clear decrease in the size of the cervical lymph nodes from the higher doses, e g. 200 mg/kg once daily decreased the mean weight from about 225 mg to 102 mg within 8 days.

Table 1 presents the results obtained after s c administration of non-lethal doses of isoprenaline There was a significant increase in the hypertrophy when the dose of isoprenaline given twice daily for 11 days, was decreased from 200 mg/kg to 100 mg/kg ($p < 0\,01$) and from 100 mg/kg to 50 mg/kg ($p < 0\,01$) When given twice daily instead of once daily 10 mg/kg was more effective but the opposite was true when the dose was 200 mg/kg ($p < 0\,01$) 20 mg/kg once daily was about as active as 50 mg/kg twice daily Subcutaneously 200 mg/kg twice daily was not lethal but clearly reduced the body weights of the animals or produced other visible toxic symptoms

Fig. 1 shows that graded responses were obtained when doses from 1 mg/kg to 100 mg/kg were injected on 7 consecutive days once daily The development of hypertrophy seems to be linear at least with doses of 20 and 100 mg/kg. Both during the first 4 days and during the subsequent 4 days of treatment with 20 mg/kg of isoprenaline the increase in weight of the salivary glands was about 1000 mg This figure was 1300 mg when the dose was 100 mg/kg. Fig. 1 also shows that when the rats were killed 3 days instead of one day after the last isoprenaline injection, the mean weights of the glands in the 20 mg/kg and 100 mg/kg

Table 1

Effect of various doses of isoprenaline on the weight of the salivary glands of rat. Means (n) ± s.e.m are given For details see text.

Duration of treatment days	mg/kg	Isoprenaline subcutaneously once a day	twice a day
11	0	516(10) ± 18	
7	10	2020(8) ± 52	2820(6) ± 166
11	20	3380(8) ± 68	
11	50		3480(6) ± 96
11	100		2630(6) ± 138
3	200		1190(3) ± 133
7	200		1870(4) ± 241
11	200	2350(6) ± 75	1600(6) ± 219

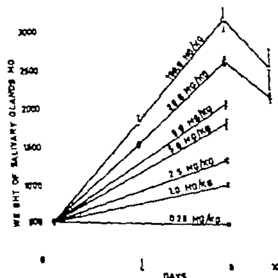


Fig. 1 Effect of 3 and 7 s. c. injections once daily of varying doses of isoprenaline on the weight of the salivary glands of rats. Means ± s.e.m. (n = from 4 to 20) are given. For details see text.

groups were, respectively 390 mg and 646 mg lower than two days before, i. e the glands had lost about 20 / and 25 / of the weight gained during the 8 days.

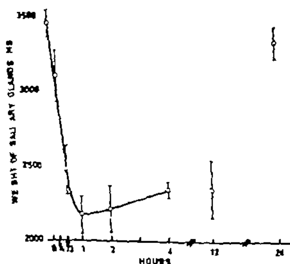


Fig. 2. Effect of 20 mg/kg of isoprenaline on the weight of hypertrophied salivary glands of rats. The animals had received 20 mg/kg of isoprenaline daily during the proceeding 11 days. Means ± s. e. m. (n = 4) are given. For details see text.

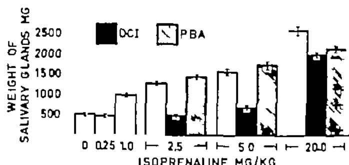


Fig. 3 Effect of 20 mg/kg of dichlorisopropylnoradrenaline (DCI) and 10 mg/kg of phenoxybenzamine (PBA) on the hypertrophy of salivary glands of rats produced by various doses of isoprenaline during 7 days Isoprenaline and PBA were given once daily and DCI twice daily Means ± s.e.m. (n = from 6 to 10) are given. For details see text

After a single s c. injection of 20 mg/kg of isoprenaline, the mean weights of the hypertrophied salivary glands were reduced from 3450 mg to 2180 mg within one hr (Fig. 2) Simultaneously there was profuse salivary excretion the glands lost their succulent appearance and they became flaccid There was no clear recovery in the size of the glands 12 hrs after the isoprenaline injection but the preinjection size was reached during the following 12 hrs

B *Effect of adrenolytics and isoprenaline*

Fig 3 demonstrates that 20 mg/kg of dichlorisopropylnoradrenaline given twice daily completely prevented the hypertrophy produced by 2.5 and 5 0 mg/kg of isoprenaline On the other hand 10 mg/kg of phenoxy benzamine given once daily had no inhibitory action

Discussion

The results reported above show that in chronic experiments, small doses of isoprenaline are more effective than large ones in causing hyper trophy of the salivary glands in the rat This is especially true when isoprenaline is injected twice daily 50 mg/kg is more effective than 100 mg/kg If given only once daily the response within 8 days increases with the dose, particularly when the dose is 20 mg/kg or less. This effect of nontoxic doses of isoprenaline has been pointed out also by WELLS (1962) and SCHNEYER (1962)

Salivary secretion after isoprenaline injection reduces the size of the enlarged glands by more than one third within one hour The replenish ment of the glandular stores occurs slowly and it is possible that the time of 24 hours after the last isoprenaline injection chosen in this study may not have been long enough to allow the glands to regain their

maximal weight, especially when the higher doses were used. It is to be noted that 7 injections of 1 mg/kg of isoprenaline once a day increase the weight of the glands by about 100 %. This dose is of the same order of magnitude as the dose used daily in sublingual tablets for the treatment of bronchial asthma. Isoprenaline hypertrophy in rats disappears within 10 to 14 days after treatment (WELLS 1962 SCHNEYER 1962)

Dichlorisopropylnoradrenaline is an adrenolytic agent capable of blocking β-receptors, the site of action of isoprenaline (POWELL & SLATER 1958) The results reported show that dichlorisopropylnoradrenaline competitively antagonizes the action of isoprenaline on the salivary glands also On the other hand, phenoxybenzamine an adrenolytic agent acting on the α-receptor sites, does not prevent the action of isoprenaline Another adrenolytic β-haloalkylamine, Dibenamine ® (dibenzylchlorethylamine), produces in high dose marked atrophy of the salivary glands of the rat (WELLS 1960) An action related to this effect may have been responsible for the small inhibitory action of phenoxybenzamine when it was given with 20 mg/kg of isoprenaline

The amputation of the lower incisor teeth of the rat leads to an enlargement of the submandibular salivary glands (WELLS *et al* 1959) Dibenamine (WELLS 1960), bretylium and reserpine (WELLS *et al* 1961) inhibit this response, which suggests that the sympathetic nervous system is involved. The hypertrophy produced by incisor amputation is also prevented by the extirpation of the superior cervical sympathetic ganglion, but the action of isoprenaline is not (WELLS 1962)

The mechanism of action of isoprenaline thus seems to be unrelated to the activity of sympathetic nerves. On the other hand, the action of noradrenaline may partly be mediated by β-receptors, because dihydroergotamine does not completely abolish the secretory effect of injected noradrenaline in rats (EMMELIN & STRÖMBLAD 1963)

Summary

The action of various doses of isoprenaline to produce salivary gland hypertrophy in rats has been studied. Isoprenaline was injected subcutaneously once or twice a day for 3 to 11 days and the parotid, submaxillary and major sublingual glands were removed 24 hrs after the last injection.

Relatively small doses were more effective than large ones in causing hypertrophy of the salivary glands. With a dose of 1 mg/kg, the total weight increase of the glands during 7 days was 91 % of the initial weight. Increasing the dose up to 100 mg/kg increased the response, but the change was most obvious with doses below 5 mg/kg.

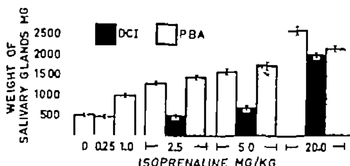


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A single injection of 20 mg/kg of isoprenaline reduced the weight of the hypertrophied glands by one third within one hour The glandular stores were replenished slowly during the next 24 hours

Dichlorisopropylnoradrenaline treatment competitively blocked the action of isoprenaline to produce hypertrophy but phenoxybenzamine was ineffective This result indicates that adrenergic β-receptors are involved in the salivary gland hypertrophy induced by isoprenaline

Acknowledgement

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Fig. 2. Fluorescent granules in cow mast cells. Magnification 1700×

mammalian chromaffin cells (Fig. 2). The fluorescence intensity also varied greatly. Most of the cells fluoresced intensively but some did so only moderately or even weakly.

The fluorescent cells showed no even distribution in the tissues. In the thyroid, heart and striated muscle, they were rather sparse and were restricted almost solely to the thicker strands of interstitial tissue and most often adjacent to the vessels. The same distribution along vessels was found in the mesentery. In the liver most of the cells were located throughout the whole capsule, although only few were found in the outer most layer. They were also abundant in the broad connective tissue septa adjacent to the capsule, but sparser in the septa within the liver and almost absent from the parenchyma. Most fluorescent cells in the lung were found in the visceral pleura and in the connective tissue septa, but a fair number of cells were situated in the interalveolar tissue.

In the duodenum, the yellow fluorescent cells were exclusively located within the epithelium, above all at the bottom of the crypts of Lieberkühn. Their cell bodies were round to pear-shaped and lay close to the basement membrane. Part of them were seen to be provided with a thin apical process that reached the epithelial surface and – like the cell bodies – contained yellow fluorescent granules. The green fluorescent cells in the duodenum occurred abundantly in the mucosa, almost solely under the

fluorescence microscope and then stained with toluidine blue (1 / in 60 ethanol) or "Astrablau" (0 1 / at pH 0.2–0.3) by the method of BLOOM & KELLY (1960). Photomicrographs of one and the same section were taken first in UV light and then, after subsequent staining, in ordinary light, so as to compare the localization of fluorescent cells and of stained cells.

The amounts of catecholamines and 5-HT in the sheep thyroid were determined by the method of BERTLER *et al* (1958) and BERTLER & ROSENGREN (1959).

Results

1 *Cow*

After treatment with formaldehyde a green fluorescence in certain granular cells and nerve fibres developed in all the tissues examined and a yellow fluorescence in scattered cells in the duodenal epithelium. If the formaldehyde gas treatment was omitted from the histochemical procedure the fluorescence did not appear

The green fluorescent cells varied greatly in shape and size In the liver capsule (Fig. 1) they were mostly rounded or angular with from one to a few short processes The dominant type in the duodenum consisted of spindleshaped forms with bipolar slender processes often of considerable length. The fluorescent material was confined to distinct cytoplasmic granules, which were larger than and not so closely packed as those in other

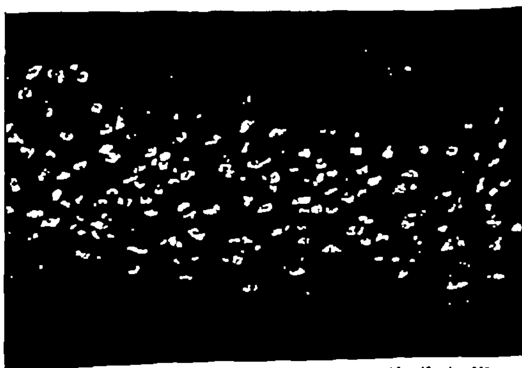


Fig. 1 Abundant fluorescent mast cells in the cow liver capsule. Magnification 250 ×



Fig. 2. Fluorescent granules in cow mast cells. Magnification 1700 x

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The fluorescent cells showed no even distribution in the tissues. In the thyroid, heart and striated muscle, they were rather sparse and were restricted almost solely to the thicker strands of interstitial tissue and most often adjacent to the vessels. The same distribution along vessels was found in the mesentery In the liver most of the cells were located throughout the whole capsule, although only few were found in the outer most layer They were also abundant in the broad connective tissue septa adjacent to the capsule, but sparser in the septa within the liver and almost absent from the parenchyma. Most fluorescent cells in the lung were found in the visceral pleura and in the connective tissue septa, but a fair number of cells were situated in the interalveolar tissue.

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epithelium cells in only moderate numbers were found in the submucosa and only few within the epithelium and in the muscular layers.

Comparison of the photomicrographs obtained of identical parts of the sections both in UV light and after subsequent staining with toluidine blue or Astrablau, revealed that the granules of the fluorescent cells were metachromatic and stained with Astrablau at pH 0 2-0 3 No other cells were found to be metachromatic or to stain with Astrablau

2. *Sheep*

In the sheep lung, liver and thyroid cells with green fluorescent granules were also seen to occur their shape and distribution were essentially the same as those seen in the cow Two noteworthy differences, however were recorded in the sheep they were much more sparse in the liver capsule and in the duodenal mucosa they also occurred in the epithelium.

In the sheep duodenum, intensely yellow fluorescent cells, most of which were located deep in the crypts of Lieberkühn, showed up in the epithelium. They were provided with a conspicuous tapering process extending to the intestinal lumen

In the thyroid, fluorescence developed in cells of two types, one emitting a green light and the other a yellow Further an intense green fluorescence developed in a relatively small number of vascular nerves. The fluorescence of the green cells was intense and clearly confined to cytoplasmic granules They were few in number compared with the yellow ones and mostly distributed along the vessels The yellow fluorescent cells occurred in great numbers but were unevenly distributed in the gland in parts of it no cells of this type could be detected Most of these cells appeared in clusters, situated next to the follicular walls but many were located within the follicular epithelium. The fluorescence of the cells, whose intensity varied considerably was clearly confined to the cytoplasm.

By comparing sections in UV light and after subsequent staining for

Table 1

Catecholamines and 5 HT in the thyroid gland of the sheep

Exper nr	5-HT µg/g	Dopamine µg/g	Noradrenaline µg/g
1	1 3	0.22	0.0
2	1 9	0.16	0 0
3	2.2	0.15	0 0
4	1 5	0.00	0.0

mast cells, as described above, it was found that only the green fluorescent cells were metachromatic and stained with Astrablau.

The results of chemical determinations of the amines are given in table 1

Discussion

The method used by us has a high specificity for certain catecholamines and tryptamines. Its sensitivity is likewise high, making possible, for example, the demonstration of arylalkylamines in neurons (CARLSSON *et al.* 1962 DAHL *et al* 1962 a FALCK 1964) where they occur in amounts too small to be detected by any other histochemical procedure, such as the chromaffin reaction (cf FALCK & HILLARP 1959) In the cow and sheep liver capsule, lung, and duodenal mucosa – submucosa, dopamine occurs in large amounts and noradrenaline only in comparatively small amounts (cf BERTLER *et al.* 1959) In the tissues from the cow and sheep a specific fluorescence showing characteristics for primary catecholamines (reaction conditions for the development of fluorescence and colour of emitted light) developed only in nerves and in a large number of granular cells. These cells show a striking correspondence in their distribution with the amounts of dopamine found in different parts of the lung, liver and duodenum (BERTLER *et al.* 1959) The fluorescence demonstrated cannot be due to 5-HT since this monoamine occurs only in small amounts in cow lung and liver capsule. Moreover the condensation product of 5-HT with formaldehyde emits a yellow fluorescence. Such a fluorescence developed in scattered intestinal cells exclusively located within the epithelium. These cells had the typical morphology of enterochromaffin cells, with a process extending to the intestinal lumen (FALCK, unpublished observation) and the typical distribution in the mucosa, with the major part located at the base of the crypts of Lieberkühn.

Thus there seems to be no doubt that the green fluorescent cells and the chromaffin cells mentioned in the introduction are the same cells, their fluorescence and their chromaffinity being due to stored dopamine.

However some discrepancies between the distribution of chromaffin cells and fluorescent cells exist. Chromaffin cells can, for example, be demonstrated only in the innermost layer of the cow liver capsule (BERTLER *et al* 1959 COUPLAND & HEATH 1961), whereas green fluorescent cells are situated throughout the capsule. Taking into consideration the well-known fact that an extraction of monoamines occurs when *liquid* fixatives are used, these discrepancies seem reasonable, especially for the most superficial parts of tissues.

The granules of the fluorescent cells – but no other structures – were found to be metachromatic and to stain in Astrablau at pH 0.2–0.3, a highly selective stain for mast cells (BLOOM & KELLY 1960) These cells must thus by definition be considered to be mast cells. This agrees with COUPLAND & HEATH (1961) who found that the chromaffin cells described by FALCK *et al* (1959 a & b) stained as mast cells in cow liver capsule and gut.

The large amounts of 5-HT found in the sheep thyroid (ERSPAMER 1961) prompted an investigation of its cellular localization, as mast cells in some species contain 5 HT The high content of 5 HT was confirmed in our investigation and it was also found that significant amounts of dopamine, but not of noradrenaline, occurred in the gland. Of the two fluorescent cell systems in this tissue, one emitted a fluorescence characteristic for primary catecholamines These cells had the typical morphology distribution pattern and staining properties of mast cells. Thus these cells must constitute the storage site of the dopamine It is most improbable that the amounts of dopamine found by the chemical determinations can be stored in the sparsely occurring adrenergic nerves. These indeed, were so sparsely distributed in the thyroid, that it entirely explains why the presence of noradrenaline could not be detected chemically

Numerous epithelioid cells – but no other structures – displayed a yellow fluorescence typical for the tryptamine group and in accordance with this, high amounts of 5 HT were found There seems to be little doubt that these cells store 5-HT They did not stain as mast cells.

The demonstration of a special cell system in the thyroid gland storing a monoamine but not belonging to the mast cell system, should be of considerable interest. A special cell system that can take up L DOPA has been demonstrated in the thyroid of other mammalian species (BERTLER FALCK & ROSENGREN unpublished results)

The presence of dopamine in ruminant mast cells should also be of biological interest So far the only species known to possess mast cells containing significant amounts of monoamines are rat and mouse whose mast cells carry 5-HT (BENDITT *et al* 1955)

Summary

1 In previous investigations a chromaffin cell system, whose chromaffinity derives from stored dopamine was reported to occur widely distributed in ruminant tissues. This cell system has now been studied by a fluorescence method highly sensitive and specific for the cellular localization of catecholamines and 5-hydroxytryptamine

2. Cells with an intense green granular fluorescence, typical for primary catecholamines, were found in all tissues examined (cow and sheep) their distribution corresponding with the occurrence of dopamine but not with that of noradrenaline. Since the fluorescence method has a sensitivity far above that of the chromaffin reaction, the cells must be identical with the chromaffin cells. However the fluorescent cells were much more numerous than the chromaffin cells and occurred also at sites where chromaffin cells have not been observed. This is not surprising, since extraction and diffusion of monoamines from their cellular stores do not occur when the technique described is used.

3. The fluorescent cells had the typical morphology, distributional pattern and staining properties of mast cells and must thus be considered to be such.

4. Cells of a new type storing 5-hydroxytryptamine, most of them having a parafollicular localization, were found in the sheep thyroid.

Acknowledgement.

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Uptake and Release of Dextro- and Laevo-Adrenaline in Noradrenergic Stores*)

By

Nils-Erik Andén

(Received February 5, 1964)

In recent years investigators have observed that circulating adrenaline (A) can be taken up and displace the normally occurring noradrenaline (NA) in sympathetically innervated organs. After administering insulin to rats, HÖKFELT (1951) found a reduction in NA and a corresponding increase in A concentration in the heart and the liver by a biological method for determination. Simultaneously the adrenals were almost completely depleted of A, which probably had been secreted into the blood and then partly taken up in sympathetic noradrenergic stores. RAAB & GIGEE (1955) injected an unusually large dose (10 mg/kg intraperitoneally) of A into dogs and found some accumulation of the administered amine in the heart. EULER (1956a) gave more moderate amounts of A to cats with a large dose (2 mg/kg i.p.) he got a small increase in A and a tendency towards a decrease in NA concentration in the heart. In the two last mentioned investigations the interval between injection and killing the animals was as short as ten minutes. STRÖMBLAD & NICKERSON (1961) injected 2.5–3 3 mg/kg A intramuscularly into rats and followed the time course of the tissue levels of NA and A. They found a considerable uptake of A and a corresponding decrease in concentration of NA in the heart and submaxillary glands. One week after excision of the superior cervical ganglion the submaxillary gland could not accumulate injected A, indicating uptake mainly by the sympathetic nerves.

There is no information in any of the above mentioned papers about whether the A used was optically active or racemic. In our study (+)-and (−)-A have been compared for their uptake in and release from the heart and the hind-leg muscles of mice. Attempts were made to obtain a

*) Results presented in part at the Meeting of the Scandinavian Pharmacological Society in Göteborg, August 24, 1962.

maximal depletion of the NA by the two A isomers in rats and mice. *Functional significance* was investigated in a few experiments. The ability of some other monoamines to deplete NA by displacement was also examined

Experiments

The comparison between the two A isomers was made on white mice weighing 20–30 g. The two isomers (as L-(+)-bitartrate salts) were dissolved in the same volumes of 0 9 / sodium chloride and injected intraperitoneally All doses of amines given in this paper are in terms of free base. After various intervals at a temperature of 28°C the animals were beheaded. The hearts and hind-leg muscles from six mice were removed. Blood was removed with filter paper The samples were homogenized in ice-cooled 0 4 N perchloric acid (3–5 ml/g tissue) After centrifugation and filtration 2 mg ascorbic acid and 20 mg EDTA (tetracemine disodium) were added to 10 ml extract in order to prevent the spontaneous oxidation that may occur on neutralization and passage through the column. The sample (pH 6.5) was put on to a Dowex 50 W column (X-4 200–400 mesh 4.2 × 50 mm Na form). The A and NA were eluted with 9 ml N HCL Differential determination of A and NA was performed spectrophotofluorometrically by the procedure of BERTLER, CARLSSON & ROSENGREN (1958). From injection to fluorometry the groups injected with (+)- and (−)- A were treated in parallel. (No differences between the two A isomers were shown by the analytical procedure employed in this study).

Between 90 and 105 / of A and NA added to tissue extracts before the column step were recovered when the proportions between the amines ranged from 1 4 to 4 1 After injecting large amounts of A, the above mentioned spectrophotofluorometric differentiation between A and NA did not give reliable NA values. For that reason a method was developed for removing A without loss of NA. An aliquot of the extract containing maximally 25 µg A was purified in the same way as described above, but the amines were eluted with 9 ml N perchloric acid, of which the first 2 were discarded. The pH of the eluate was adjusted to 3.5 (indicator paper) with 5 N potassium carbonate. After cooling to 0° the potassium perchlorate was spun down in a refrigerated centrifuge. The supernatant was transferred to an Erlenmeyer flask and 0.5 ml M sodium acetate buffer pH 3 55 0 1 ml 0.5 / zinc sulphate and 1 0 ml 25 / potassium ferricyanide were added. After the mixture had stood for 30 minutes at room temperature, the oxidation was interrupted by adding 1 0 ml 25 / ascorbic acid. (By these procedures the A was completely oxidized to adrenochrome and the NA was unchanged. The oxidation was inhibited by high ionic concentrations but these could be kept low by using perchloric acid for elution and subsequent precipitation of potassium perchlorate (solubility at 0°C 7 5 g/l)). The sample was again put on to a Dowex 50 column at a rate of 0.5 ml/min. After washing twice with 20 ml redistilled water the NA was eluted with 9 ml N HCl. (The adrenochrome passed freely through the cation exchange column. The NA was quantitatively taken up by the ion exchanger although the amount of cations exceeded the capacity of the column 3–4 times. The recovery of NA from tissue homogenates varied between 80 and 100 . No corrections for incomplete recovery have been made.)

Paper chromatography (butanol N HCl, 4 1) was used for semiquantitative determination of adrenalone in the rat heart after injection of this amine The extraction and purification was performed as described above, but the amines were eluted with

9 ml N-HCl and then by 9 ml 2 N-HCl. The first 2 ml N-HCl were discarded. The paper chromatography was performed as described by CARLSSON & LINDQVIST (1962). Afterwards the paper was dried and sprayed with 0.1 % potassium ferricyanide in 5 % ethylene diamine, dried for five minutes at 50–60° and examined for fluorescence in UV light (ELLMAN 1958).

The ß-O-methyl NA was extracted as described above. The sample (pH 6.5) was passed through a long Dowex 50 W X4 column (4.2 × 90 mm at pH 0) buffered at pH 6.5 (CARLSSON & WALDECK 1963). After washing with 60 ml water the elution was done with HCl. The first 8 ml N-HCl were discarded, and the next 9 ml N-HCl were collected for determination of NA. The next 20 ml 2 N-HCl contained the ß-O-methyl NA. The last eluate was reduced in volume to one drop in a rotating evaporator. The solution became strongly acid, and in these circumstances the ß-O-methyl NA was transformed into NA quantitatively. After addition of a suitable amount of N-HCl, the final determination was performed as described above for NA.

The dopamine was determined by the method of CARLSSON & WALDECK (1958) as modified by CARLSSON & LINDQVIST (1962). The 5-hydroxytryptamine was determined as described by BERTLER (1961), except that the tissue residue was re-extracted once with the original volume of 0.4 N perchloric acid (5 ml/g tissue).

Results

Comparison between (+)-and (–)-adrenaline

Adrenaline rapidly appeared in the mouse heart after an intraperitoneal injection of 2 mg/kg of either (+)- or (–)-A (fig. 1). The accumulation of A was somewhat greater after the (–) than after the (+) isomer. After about two hours the A concentration began to decrease at about the same rate with either isomer. The half life of A may be roughly estimated at 12 hours. Simultaneously with the A uptake the NA concentration of the heart dropped in 90 minutes by approximately 60 % after the (+)-and 75 % after the (–)-A from the normal 5.2 nmol/g (0.87 μg/g; 1 nmol = 10^{-9} gmol; s.e.m. = 0.37 nmol/g, 6 experiments). After

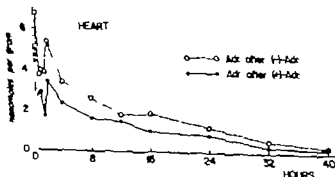


Fig. 1. Levels of adrenaline in mouse heart after 2 mg/kg (+)- or (–)-adrenaline intraperitoneally.

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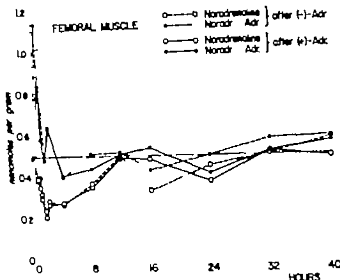


Fig. 4 Levels of noradrenaline and total catecholamines (noradrenaline + adrenaline) mouse hind-leg muscles after 2 mg/kg (+)- or (−)-adrenaline intraperitoneally The dotted line indicates the normal concentration of noradrenaline + adrenaline.

ments) in 90 minutes (fig. 4). Again the NA depletion was balanced by accumulation of A (see fig. 4 A + NA normally 0.50 nmol/g with s.e.m. = 0.014 nmol/g, 6 experiments) The accumulated A disappeared and the NA level rose more rapidly than in the heart. The half life of A was estimated at eight hours and was about the same after either isomer As in the heart there was a tendency towards a larger uptake of A and a larger depletion of NA after the (−) isomer but the difference between the enantiomorphs was somewhat smaller in the hind-leg muscles.

In normal mice the catecholamines of the heart and hind-leg muscles were completely depleted 18 hours after administering reserpine (10 mg/kg i.p.) When (+)- or (−)-A (2 mg/kg i.p.) was given to such animals two hours before they were killed, no A was accumulated in these tissues.

The ability of reserpine to deplete the accumulated A was investigated in one experiment. Four hours after the injection of the A isomers (2 mg/kg i.p.) reserpine (10 mg/kg i.p.) was given and the mice were killed two hours later Reserpine reduced both A and NA concentrations in the heart and hind leg muscles to between 25 and 40 / of those in controls that had received only A. The reserpine-induced reduction in A concentration was about the same after administration of either A isomer

After a single injection as well as with increasing doses of A the symp-

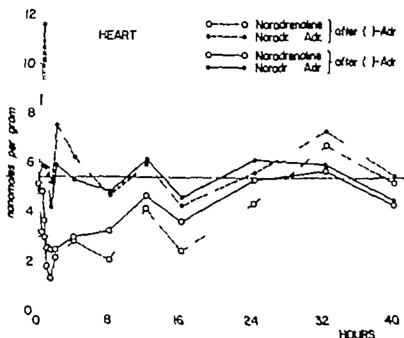


Fig. 2. Levels of noradrenaline and total catecholamines (noradrenaline + adrenaline) in mouse heart after 2 mg/kg (+)- or (−)-adrenaline intraperitoneally. The dotted line indicates the normal concentration of noradrenaline + adrenaline.

the first few hours the depletion of NA was almost exactly balanced by the accumulation of A (see fig. 2, A + NA. A + NA normally 5.4 nmol/g with s.e.m. = 0.38 nmol/g, 6 experiments).

In the hind leg muscles A was also taken up, but the concentration of A, like the normal concentration of NA, was about ten times lower than in the heart (fig. 3). The NA concentration was reduced by some 60–70 % of the normal 0.49 nmol/g (0.083 µg/g; s.e.m. = 0.014 nmol/g, 6 experi-

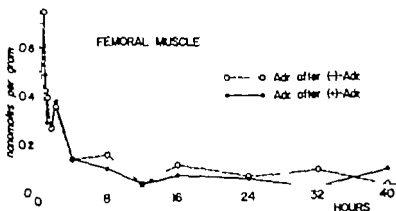


Fig. 3. Levels of adrenaline in mouse hind-leg muscles after 2 mg/kg (+)- or (−)-adrenaline intraperitoneally.

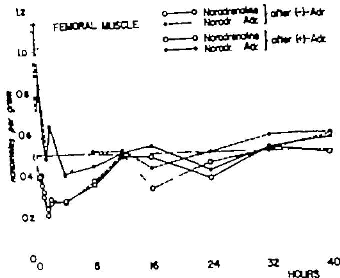


Fig. 4. Levels of noradrenaline and total catecholamines (noradrenaline + adrenaline) in mouse hind-leg muscles after 2 mg/kg (+)- or (−)-adrenaline intraperitoneally. The dotted line indicates the normal concentration of noradrenaline + adrenaline.

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After a single injection as well as with increasing doses of A the symp-

toms appeared in the order piloerection exophtalmos, salivation (large individual differences) hyperpnoea obducted hind legs. The two A isomers gave the same symptoms qualitatively but the (+) form had to be given at an approximately 20 times larger dose than the (−) to produce the same quantitative effects. When 2 mg/kg (−)-A were injected intraperitoneally the symptoms appeared 3–5 minutes after the administration and lasted for 1–2 hours The same dose of (+)-A produced no detectable symptoms. Reserpine treatment (10 mg/kg i p 16 hours earlier) did not abolish the symptoms due to (+)- or (−)-A indeed, they both seemed to be enhanced somewhat

Severe noradrenaline depletion by the two adrenaline isomers

Dextro-adrenaline These experiments were on hooded rats (inbred strain 200–300 g) because functional studies were easier to perform on them than on mice The rats were injected intraperitoneally with 40 mg/kg (+)-A. They were kept at a temperature of 28 C The concentration of NA in the heart decreased from the normal 5 1 nmol/g (0 85 µg/g s e.m. = 0 48 nmol/g, 3 experiments) to 2.5 in two 1 2 in four and 1 0 nmol/g in six hours (fig. 5) The A concentration decreased from 40 nmol/g after

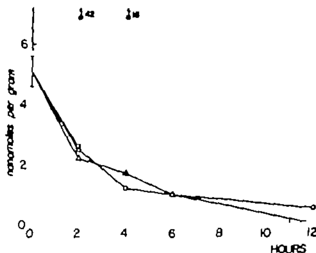


Fig. 5 The depletion of noradrenaline in rat heart caused by reserpine (10 mg/kg i.p.), (+)-adrenaline (40 mg/kg i.p) o (+)-adrenaline (2 mg/kg i p). The normal value (indicated on vertical axis) is an average of 3 determinations ± s e.m

△———△ Noradrenaline after reserpine (10 mg/kg) at time 0.
○———○ Noradrenaline after (+)-adrenaline (40 mg/kg) at 0 and 6 hours.
● Noradrenaline + adrenaline after (+)-adrenaline (40 mg/kg) t 0 and 6 hours.
□———□ Noradrenaline after (+)-adrenaline (2 mg/kg) at time 0.
■ Noradrenaline + adrenaline after (+)-adrenaline (2 mg/kg) at time 0.

two hours to 4.4 nmol/g after six hours, *i. e.* to a level approximately corresponding to the amount of missing NA. If (+)-A was given at the same dose six hours after the first injection, the NA concentration of the heart decreased to 0.52 nmol/g six hours later (fig. 5). At that time the A concentration was 4.9 nmol/g, *i. e.* the total catecholamine concentration was about normal. In a rat similarly treated the cervical sympathetic was cut unilaterally under pentobarbital sodium anesthesia (30 mg/kg i.p., Nembutal-Abbott for veterinary use) four hours after the second injection. Two hours later the rat was awake again. There was a distinct ptosis on the denervated side, indicating no eye effects of circulating A, and the eye-lid on the intact side was normally elevated. The NA and A concentrations of the heart were of the same order of magnitude as in the unoperated rat. Thus, there seemed to be no severe disturbance of adrenergic transmission after displacement of approximately 90 % of the NA by the much less active (+)-A.

When 2 mg/kg (+)-A were given intraperitoneally to a rat, the NA concentration of the heart dropped in two hours to 2.6 nmol/g or to about the same level as after 40 mg/kg (fig. 5). The A concentration was 2.4 nmol/g.

After an intraperitoneal injection of 40 mg/kg (+)-A the NA concentration of the rat adrenals dropped from 0.24 μmol/kg body weight (50 μg/kg body weight, s.e.m. = 0.031 μmol/kg, 3 experiments) to 0.17 in six hours. The difference was, however, not significant. The A concentration was at the same time essentially unchanged or 1.10 μmol/kg body weight against normally 1.04 (190 μg/kg body weight, s.e.m. = 0.080 μmol/kg, 3 experiments).

Two hours after the injection of (+)-A (40 mg/kg i.p.) the NA concentration of the rat brain was 2.4 nmol/g, which did not differ significantly from the normal (2.6 nmol/g = 0.44 μg/g, s.e.m. = 0.20 nmol/g, 3 experiments). The A concentration at the same time was 0.76 nmol/g, and the heart contained 40 nmol/g.

The drop of NA in the rat heart and adrenals caused by reserpine (10 mg/kg i.p.) was compared with that caused by (+)-A (fig. 5). Reserpine reduced the NA level of the heart from 5.1 nmol/g to 2.2 in two, to 1.7 in four and to 1.0 nmol/g in six hours. In the adrenals reserpine reduced the NA concentration faster than (+)-A. After six hours the NA concentration had decreased from 0.24 to 0.09 μmol/kg body weight, and the A level had dropped in the same proportion.

Laevo-adrenaline. This experiment was made on white mice (20–30 g) because they tolerated (−)-A better than rats. The (−)-A was injected intraperitoneally six times (2 mg/kg/dose) at intervals of two hours, and the animals were beheaded 1½ hour after the last injection. The NA level

toms appeared in the order piloerection, exophtalmos, salivation (large individual differences) hyperpnoea obducted hind legs. The two A isomers gave the same symptoms qualitatively but the (+) form had to be given at an approximately 20 times larger dose than the (−) to produce the same quantitative effects. When 2 mg/kg (−)-A were injected intraperitoneally the symptoms appeared 3–5 minutes after the administration and lasted for 1–2 hours. The same dose of (+)-A produced no detectable symptoms Reserpine treatment (10 mg/kg i.p 16 hours earlier) did not abolish the symptoms due to (+)- or (−)-A indeed they both seemed to be enhanced somewhat.

Severe noradrenaline depletion by the two adrenaline isomers

Dextro-adrenaline These experiments were on hooded rats (inbred strain 200–300 g) because functional studies were easier to perform on them than on mice. The rats were injected intraperitoneally with 40 mg/kg (+)-A They were kept at a temperature of 28 C The concentration of NA in the heart decreased from the normal 5 1 nmol/g (0 85 µg/g s.e.m. = 0 48 nmol/g, 3 experiments) to 2 5 in two 1 2 in four and 1 0 nmol/g in six hours (fig 5) The A concentration decreased from 40 nmol/g after

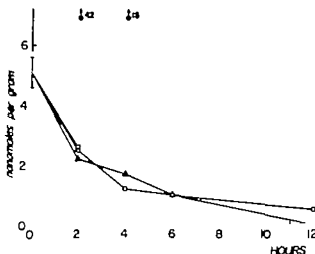


Fig. 5 The depletion of noradrenaline in rat heart caused by reserpine (10 mg/kg i p.), (+)-adrenaline (40 mg/kg i p) or (+)-adrenaline (2 mg/kg i.p) The normal value (indicated on vertical axis) is an average of 3 determinations ± s.e.m

△———△ Noradrenaline after reserpine (10 mg/kg) at time 0
○———○ Noradrenaline after (+)-adrenaline (40 mg/kg) at 0 and 6 hours.
● Noradrenaline + adrenaline after (+)-adrenaline (40 mg/kg) at 0 and 6 hours.
□———□ Noradrenaline after (+)-adrenaline (2 mg/kg) at time 0.
■ Noradrenaline + adrenaline after (+)-adrenaline (2 mg/kg) at time 0.

Table 1

Levels of monoamines after injection of mines or amine precursors. NA = noradrenaline. All results in mol/g.
The normal concentrations of noradrenaline (verage ± s.e.m = number of determinations) l nmol/g at heart 5 1 ± 0.48 (= 3), mouse heart 5.2 ± 0.37 (= 6), mouse brain 2.6 ± 0.15 (= 3).

Injected compound	Dose and time	Organ	NA	NA deficit	Other monoamines
Adrenalone	40 mg/kg i p 2 h 40 mg/kg p., 6 h 2 mg/kg p., 2 h	Rat heart Rat heart Rat heart	2.2 2.4 4.7	2.9 2.7 0 4	Adrenalone = 5.5) Adrenalone = 2.0) Adrenalone not determined
β-O-methyl NA	40 mg/kg i.p., 2 h 40 mg/kg i p., 6 h 2 mg/kg i p., 2 h	Rat heart Rat heart Rat heart	2.4 3 5 4.1	2.7 1 6 1.0	β-O-methyl NA = 12 β-O-methyl NA = 0.9 β-O-methyl NA not determined
Epinine	40 mg/kg i.p., 2 h	Mouse heart	2.0	3.2	Adrenaline = 0.4 Epinine not determined
Dopamine	2 mg/kg i.p., 2 h	Mouse heart	7 7	−2.5	Dopamine = 0
5-hydroxytryptamine	500 mg/kg i.p., 2 h 2 mg/kg i.p., 2 h	Mouse heart Mouse heart	2.2 4.2	3.0 1.0	5-hydroxytryptamine = 160 5-hydroxytryptamine not determined
DL-5-hydroxytryptophan	500 mg/kg i p., 2 h	Mouse heart Mouse brain	2.5 1.6	2.7 1.0 (dopamine deficit = 1.5 nmol/g)	5-hydroxytryptamine = 22 5-hydroxytryptamine l excess = 9 7

) Determined on paper chromatograms.

of the heart was reduced from 5 2 to 0 95 nmol/g. The A concentration was 9 8 nmol/g. The animals appeared somewhat apathetic but otherwise normal at the time they were killed

Depletion of noradrenaline by other noradrenaline analogues

Adrenalone The NA concentration of the rat heart was reduced to about half of the normal at two and at six hours after an intraperitoneal injection of adrenalone in a dose of 40 mg/kg (table I) At both intervals a fluorescent spot with the same colour (yellow green) and position (Rf = 0.26) as those of authentic adrenalone was observed by paper chromatography The amounts were estimated at about twice as large at two hours and about the same as the lost NA at six hours after the injection. No A formation from the administered adrenalone could be detected after either interval by means of spectrophotofluorometry or paper chromatography It, thus seems as if adrenalone causes displacement of the NA in the heart without being reduced to A. Adrenalone is probably a less potent displacer than A, since there was no significant decrease of NA level in the rat heart two hours after 2 mg/kg intraperitoneal adrenalone (table I)

β-O-methyl noradrenaline Like A and adrenalone β-O-methyl NA (racemic, H 22/38) at a dose of 40 mg/kg intraperitoneally reduced the NA concentration of the rat heart to about half the normal in two hours (table I) It increased somewhat between two and six hours At the earlier interval the concentration of β-O-methyl NA much exceeded that of the missing NA, whereas at the later the two were approximately the same. An intraperitoneal injection of 2 mg/kg β-O-methyl NA produced only a slight reduction of the NA concentration in the rat heart (table I)

Epinine Also when epinine (N methyldopamine) was given in a dose of 40 mg/kg intraperitoneally to mice, the NA of the heart was depleted in two hours to a level somewhat lower than half the normal (table I). Simultaneously an amount A corresponding to between 10 and 20% of the missing NA was detected spectrophotofluorometrically No determination of epinine was made

Dopamine Two hours after an intraperitoneal injection of dopamine at a low dose (2 mg/kg) there was no reduction of the NA concentration in the mouse heart (table I) Indeed the NA level was increased by approximately 50% probably on account of formation of NA from the dopamine administered No dopamine could be detected in the heart after this time.

5 Hydroxytryptamine As seen in table I the NA concentration of the mouse heart was reduced to between 40 and 50% of the normal two hours after a large dose of 5-hydroxytryptamine (500 mg/kg i p) or its precursor

indicate that a methyl group on the nitrogen atom does not prevent uptake in the noradrenergic stores and a displacement of the naturally occurring transmitter. Unpublished experiments with isoprenaline show that the same is valid also for the isopropyl group, but that the uptake is markedly reduced. The hydroxyl group on the β-carbon atom of (−)-NA does not seem necessary for uptake, to judge from the experiments with the two A isomers, adrenalone, β-O-methyl NA, dopamine and epinine. It seems, however, as if (+)-A is taken up to a somewhat smaller extent than (−)-A, so that there is probably some stereospecificity in this process. The investigations with a small dose (2 mg/kg i.p.) of adrenalone and β-O-methyl NA show that a keto or methoxy group on the β-carbon atom presents a much larger obstacle than the hydroxyl group of (+)-A. It is impossible to tell from the results presented here whether or not dopamine can displace the NA in sympathetic nerves. With higher doses of dopamine, however, HARRISON, LEVITT & UDENFRIEND (1963) were able to get depletion of the NA from the guinea pig heart, results supporting the hypothesis that the NA depletion after epinine also is mainly due to a displacement by the injected compound. Epinine is a rather poor substrate for β-hydroxylase (GOLDSTEIN & CONTRERA 1962, CREVELING *et al.* 1962). This fact probably explains why there is only a slight formation of A from epinine. Also, compounds with a methyl group on the α-carbon atom can displace NA, which occurs with α-methyl NA and metaraminol injected as amines or formed from their amino acid precursors (CARLSSON & LINDQVIST 1962, CARLSSON 1963, MAÎTRE & STAEHELIN 1963, ANDÉN, unpublished). In fact, these agents accumulate after smaller doses and remain for a longer time in the tissues than A, probably because they are not easily degradated by monoamine oxidase. The uptake of 5-hydroxytryptamine has been observed in many sympathetically innervated organs and in the adrenals after an injection of the labelled compound (AXELROD & INSCOE 1963). By means of a specific histochemical method it has been observed that the pineal nerves of the rat normally take up 5-hydroxytryptamine formed in the pinealocytes (FALCK & OWMAN, personal communication). After an injection of 5-hydroxytryptophan into rabbits, BERTLER, ROSENGREN & ROSENGREN (1960) observed an uptake of 5-hydroxytryptamine in the granular fraction of the adrenal medulla. The uptake of 5-hydroxytryptamine seemed to be smaller than that of the A isomers, judged from the extent of NA depletion after an injection of 2 mg/kg.

It has been found that the uptake of labelled catecholamines into isolated storage granules from the adrenal medulla is inhibited twice as much after addition of (−)- than of (+)-A (CARLSSON, HILLARP & WALDECK 1963). The discrepancy in results between these *in vitro* experi-

5-hydroxytryptophan (500 mg/kg of the DL form) In both instances large amounts of 5-hydroxytryptamine were found in the heart. There was also some depletion of the brain NA after the injection of 5-hydroxytryptophan (table 1) The NA depletion of the heart was much less after 2 mg/kg 5-hydroxytryptamine than after the same dose of A.

Discussion

It is important to know where the accumulated A is localized in the tissues. The A concentration is after all intervals ten times greater in the heart than in the hind leg muscles, the same ratio as between normal NA concentrations in these tissues This argues against a diffuse distribution, for example in the extracellular fluid, or any unspecific binding. The accumulated A corresponds almost exactly to the missing NA in the tissues *This indicates that the A is taken up in the noradrenergic stores of* the sympathetic nerves STRÖMBLAD & NICKERSON (1961) arrived at the same conclusion from the fact that no A accumulated in the submaxillary gland one week after excision of the superior cervical ganglion. The NA in the tissues is mainly localized in sympathetic nerve terminals, in which it probably occurs in storage granules, as in the adrenal medulla and the sympathetic nerve trunks (for references, see EULER 1956b) The mode of action of reserpine seems to be by inhibition of active incorporation of monoamines into these storage granules (KIRSHNER 1962 CARLSSON, HILLARP & WALDECK 1962 EULER & LISHAJKO 1963a) The results showing that reserpine releases A and NA equally and also inhibits the uptake of A give further support to the assumption that the accumulated A is localized to the noradrenergic storage granules in sympathetic nerves.

The two isomers of A are taken up to approximately the same extent in the heart and hind leg muscles. It may be argued that the injected and accumulated A does not rotate plane polarized light in the same manner A conversion to the isomer may occur by racemization or possibly by oxidation to adrenalone and subsequent reduction to adrenaline It is unlikely that such conversions occur before the amines enter the sympathetic nerves, as the pharmacological effects after injections of the two isomers differ quantitatively Racemization certainly does not take place to any significant degree in the body as naturally occurring A and NA are entirely laevorotatory (for references, see EULER 1956b) The experiments showing that adrenalone causes displacement of NA without being reduced to A, seem to exclude the possibility that one A isomer is converted to the other with adrenalone as an intermediate

The results obtained after the injection of A, adrenalone or epinine

the total catecholamine concentration is normalized after about six hours. The NA concentration of the heart decreases at approximately the same rate after (+)-A and reserpine (see fig. 5). This fact and the dose independent depletion of NA by (+)-A may indicate that the reduction in NA concentration by both these drugs is due to an interference with a normally occurring movement of NA. Reserpine is known to inhibit active incorporation of monoamines into the storage granules, which may explain its amine-depleting action (references, see above). It has been observed that A and reserpine compete for the same groups in the uptake mechanism for monoamines in the adrenal medullary granules (JONASSON, ROSENGREN & WALDECK 1963). Thus, the NA depleting effect by A may occur at the same point of attack as reserpine, but also other steps of the NA uptake may possibly be inhibited. Simultaneously A is taken up, so that it completely balances the NA loss. During the first hours even a little more A is accumulated than corresponds to the missing NA. The A in excess may possibly be localized in a more "labile" fraction, as it rapidly disappears. Later on, all the A is found in the more "stable" fraction, where most of the normally occurring NA is present. During this period the A concentration almost exactly corresponds in amount with that of the missing NA. MUSCHOLL (1960) found that, after an intravenous infusion of 20 μg NA, the normal rat heart took up excess NA, which decreased to half in 40 minutes. After reserpine treatment the same infusion gave no NA accumulation at all, indicating that the "labile" fraction also is localized in the granules. In fact, it has been found that about 20 % of the catecholamines in the adrenal storage granules are not bound to adenosine triphosphate and are liberated more easily into the supernatant at centrifugation (HILLARP 1960 a).

It has been found in this laboratory that labelled A and NA (racemic forms), accumulated in tracer amounts in the heart or hind-leg muscles, disappear at about the same rate four hours after the injection (WALDECK, unpublished). This indicates that the above-mentioned halflives of the two A isomers in the heart and hind-leg muscles may approximately correspond to those of the naturally occurring NA. The half-lives of the radioactive A are of the same order of magnitude as those reported in this paper

The spontaneous elimination of accumulated A seems to occur approximately six times more slowly than the depletion of normally occurring NA by reserpine or A. As discussed above, these drugs may interfere with the uptake of NA, yet leave spontaneous release from the granules unaffected. If the block of uptake is complete, these results indicate that about ⅚ of the amines normally released from the »stable« fraction of

ments and the investigations reported above may be explained if the (+)-reaches the tissues in question in larger amounts than (−)-A. The cause of such a difference could be that the two A isomers are not metabolized equally The catechol-O-methyl transferase from rat liver is reported to metabolize the two isomers equally *in vitro* (AXELROD & TOMCHICK 1958). Also monoamine oxidase seems to degrade the isomers about equally *in vitro* (BLASCHKO RICHTER & SCHLOSSMANN 1937 LEEPER, WEISSBACH & UDENFRIEND 1958) After oral administration of (+)- and (−)-A at the same doses, the two isomers are excreted in the urine as conjugates in high and about equal amounts (RICHTER 1940) but this metabolic pathway is probably of importance only for this route of administration (HÄGGENDAL 1963) Though the two A isomers *in vitro* seem to be attacked equally by all known enzymes, they may differ in their metabolism *in vivo* if they do not reach the inactivating enzymes in equal amounts. After an intraperitoneal injection much of the A seems to be degraded by the liver catechol-O-methyl transferase (CARLSSON & WALDECK 1963), and the liver cells could predominantly take up the (−)-A, as did the adrenal medullary granules in the experiments mentioned above. The (−)-A isomer should also be taken up by the liver cells to a larger extent than (+)-A if it produces greater vasoconstriction in the liver In preliminary experiments it was found that the blood concentration of A at all intervals was larger after the (+)- than after the (−)- isomer The hypothesis that a lesser degradation of (+)-A is responsible for the about equal uptake of the two A isomers is supported by results from experiments with the two isomers of metaraminol. As is well known, this substance seems to be metabolized slowly It has been found that the NA depletion caused by the (+)- metaraminol (H 22/22) was much smaller than that caused by the (−)- isomer (unpublished experiments) The discrepancy between the *in vivo* and *in vitro* results may also be explained by local circulatory changes in the tissues investigated. The possibility may even be considered that the two isomers influence local blood flow in opposite directions There is also the possibility that the almost equal uptake of the two A isomers after the dose used is due to high intraneuronal concentrations, as it has been found that the uptake into isolated granules from sympathetic nerves is stereospecific only at low external concentrations of catecholamines (EULER & LISHAJKO 1963b)

The NA concentration of the heart drops to about the same value over two hours both after 2 and 40 mg/kg (+)-A (see fig. 5) The A concentration after the smaller dose then corresponds to the missing NA whereas it is much higher after the larger dose This probably explains why the drop in NA level continues after the larger dose and does not cease until

whereas the pupil size as well as the position of the nictitating membrane on the intact side did not deviate significantly from those of normal animals. Thereafter the cats were anesthetized with pentobarbital sodium (30–40 mg/kg i.p.) and the sympathetic nerves were stimulated in different ways. When the cervical sympathetic was stimulated preganglionically (6 V 10 msec., 1–10 imp./sec.), the pupil dilated and the nictitating membrane contracted as in control animals. The blood-pressure responses to tyramine (0.5 mg/kg) and splanchnic nerve stimulation (10 V 10 msec., 10–20 imp./sec.) were also in the normal range, even after bilateral adrenalectomy The NA of the heart, spleen, iris and nictitating membrane was displaced to approximately 95 / by (+)-A, *i e* the adrenergic transmission seemed to operate even when the biological activity of the stored transmitter had been reduced about ten times. The identical disappearance rates of (+)- and (−)-A also support the assumption that most of the store of adrenergic transmitter is not essential for normal transmission.

Summary

After intraperitoneal injection of 2 mg/kg (+)- or (−)-adrenaline into mice, the noradrenaline of the heart and hind-leg muscles was displaced to the extent of about 50 / by adrenaline. Reserpine blocked the uptake of adrenaline and depleted the accumulated adrenaline. The noradrenaline of the rat heart dropped at the same rate, after either 2 mg/kg or 40 mg/kg dextro-adrenaline and after a large dose of reserpine. Adrenaline gave no depletion of the noradrenaline in the brain and only an insignificant reduction in the adrenals. After repeated injections of (+)- and (−)-adrenaline the noradrenaline of the heart was displaced to the extent of 90 and 80 / of the normal, respectively There was no detectable disturbance of adrenergic transmission after this severe noradrenaline displacement by the about ten times less active (+)-adrenaline. The two adrenaline isomers disappeared from the mouse tissues at about equal rates, with estimated half-lives of 12 and 8 hours in the heart and hind-leg muscles, respectively Injections of adrenalone, β-O-methyl noradrenaline or 5-hydroxytryptamine also caused a noradrenaline depletion and an uptake of the administered amine.

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the granules are taken up again. The ability of the sympathetic nerves to take up injected amines in large quantities may suggest that re-uptake is an important way of preserving the transmitter

Whereas A and reserpine seem to reduce NA concentration at about the same rate in sympathetically innervated organs, the other noradrenaline stores of the body are depleted to a much smaller extent by A than by reserpine In brain there is no detectable decrease in NA level and a small accumulation of A compared with that in the heart, probably on account of the poor penetration of A through the lipid like blood brain barrier It is possible that the small amount of accumulated A in brain is mainly localized in vasomotor nerves and in the lumen of the vessels. In the adrenals, reserpine depletes at least twice as much NA as does a large dose of (+)-A in six hours. Reserpine seems to deplete the amines of the adrenal medulla in two ways first, by a direct effect on the adrenal storage granules and, secondly by an indirect effect mediated by an increased impulse flow in the splanchnic nerves (HILLARP 1960b) The insignificant uptake of (+)-A may be due to a lack of stimulation of the adrenal medullary cells in which probably the turnover rate is normally low There is, however the possibility that the adrenal medulla cannot accumulate extracellular amines. The explanation of the uptake in the sympathetic nerve endings may possibly be a rapid physiological inactivation of the transmitter The adrenal medulla has clearly no need of such a mechanism The adrenals seem however to be able to take up labelled adrenaline (AXELROD WEIL MALHERBE & TOMCHICK 1959)

The similarity in uptake of the two A isomers contrasts with the difference in receptor stimulation. Judged from the appearance of the mice, the pharmacological effect of the (+)-A is about 20 times weaker than that of the (−)-A. Approximately the same difference was found in blood pressure responses (both decreases and increases) of normal cats and rats (ANDÉN & MAGNUSSON unpublished experiments) After reserpine treatment, this difference in activity remained unchanged. This fact indicates that the effects of the (+)-isomer are a direct receptor stimulation rather than a liberation of NA from the nerve terminals.

The adrenergic innervation of the rat eye is apparently unimpaired when the NA in the sympathetic nerves is displaced by the much less active (+)-A, as judged by the lack of ptosis The functional significance of this displacement was further studied in cats (ANDÉN & MAGNUSSON unpublished) The cats had usually undergone unilateral cervical sympathectomy so as to check the sympathomimetic effects of circulating amines. Six hours after the first of the two large intraperitoneal injections of (+)-A, each 20 mg/kg after an interval of two hours, there was miosis and relaxation of the nictitating membrane on the decentralized side,

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Swedish Ciba, Stockholm Sweden (reserpine) Dr H Corrodi, Hässle Ltd Göteborg, Sweden (β-O-methyl noradrenaline, (+)-metaraminol) and Dr K C. Mezey Merck Sharp and Dohme Research Laboratories, Rahway New Jersey U S A. ((−)-metaraminol) For technical assistance I am grateful to Miss Birgitta Nilsdotter Högberg.

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washed erythrocytes were made by a modification of Hestrin's method (FLEISHER & POPE 1954). The material included blood samples from 130 dead bodies, but no samples from live subjects, as the method chosen enabled the investigators to use Fleisher & Pope's normal values.

In both sets of investigations the material was divided up into different groups according to sex, age, race and cause of death and also according to the individual parts of the body from which the blood samples were taken. Minor but insignificant, differences between the groups were demonstrated within both sets of investigations. No differences were found between the activity of blood samples from live subjects and that of post-mortem blood samples. The investigations made by Pribilla comprised 11 deaths caused by phosphostigmine poisoning, whereas no blood samples from individuals killed by phosphostigmine were included in Petty *et al*'s material. Both studies showed that phosphostigmine poisoning involves a considerable decrease of cholinesterase activity.

In discussing autopsy samples from individuals who did not die from phosphostigmine poisoning, both PRIBILLA and PETTY *et al.* conclude that the cholinesterase activity of separated plasma and erythrocytes can both be characterized by a mean value and its range.

The above mentioned routine determinations of cholinesterase activity were performed in the forensic chemical section of our institute by a modification of Michel's method (HUANO 1955), and the investigations were made on whole blood.

The object of our work was to obtain more comprehensive control material for the cholinesterase activity of autopsy blood determined by automatic titration, with a view to answering three questions: 1) Is erythrocyte cholinesterase activity a better criterion than plasma cholinesterase activity for detecting a phosphostigmine poisoning? 2) What is the applicability of various choline esters as substrates in such examinations? 3) How important is the haemoglobin concentration for the evaluation of cholinesterase activity in whole blood from dead bodies?

The following *symbols* will be used:

AChE erythrocyte cholinesterase, acetylcholinesterase.

BuChE plasma cholinesterase, butyrylcholinesterase.

ChE cholinesterase, not specified.

The symbols in brackets indicate the enzyme activity. The following substrate designations are used: ACh for acetylcholine, MeCh for methacholine (acetyl-β-methylcholine) and BuCh for butyrylcholine.

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Cholinesterase Activities of Human Autopsy Blood with Special Reference to Phosphostigmine Poisoning

By

H H Lausen

(Received February 4 1964)

During recent years a vastly increasing amount of pesticides has been employed. This holds especially for cholinesterase inhibiting insecticides, a large group of what are generally known as organic phosphorus insecticides etc. In what follows they will be named *phosphostigmines* (stigmine" after physostigmine) (MØLLER JENSEN-HOLM & LAUSEN 1961) Most phosphostigmines are extremely poisonous to humans and domestic animals forensic medicine and chemistry present a clear picture of their effects The application of phosphostigmines will result in irreversible inhibitions of the cholinesterases of the organism in toxicological investigations determination of the cholinesterase activity of blood will therefore be a valuable indicator of any possible poisoning with these compounds. Since 1955 such routine investigations have been made in this laboratory in connection with forensic chemical examinations. The value of such investigations seems, however not to be widely recognized. We have found only two studies reporting determinations of the cholinesterase activity in a considerable number of autopsy blood samples (PRIBILLA 1957 PETTY LOVELL & MOORE 1958)

PRIBILLA determined solely the activity of the plasma cholinesterase by a modification of MICHEL s electrometric method (1949) the pH displacement being determined photometrically with phenol red as an indicator Haemoglobin free plasma was used for these examinations and from several samples Pribilla succeeded in separating the plasma up to 72 hours after death. Pribilla s material consisted of blood samples from 60 dead bodies and 112 live subjects

PETTY and coworkers only determined the activity of the erythrocyte cholinesterase, which they considered a better subject for investigation than the plasma cholinesterase The investigations of separated, but un

washed erythrocytes were made by a modification of Hestrin's method (FLEISHER & POPE 1954). The material included blood samples from 130 dead bodies, *but no samples* from live subjects, as the method chosen enabled the investigators to use Flesher & Pope's normal values.

In both sets of investigations the material was divided up into different groups according to sex, age, race and cause of death and also according to the individual parts of the body from which the blood samples were taken. Minor but insignificant, differences between the groups were demonstrated within both sets of investigations. No differences were found between the activity of blood samples from live subjects and that of post-mortem blood samples. The investigations made by *Pribilla* comprised 11 deaths caused by phosphostigmine poisoning, whereas no blood samples from individuals killed by phosphostigmine were included in Petty *et al*'s material. Both studies showed that phosphostigmine poisoning involves a considerable decrease of cholinesterase activity

In discussing autopsy samples from individuals who did not die from phosphostigmine poisoning, both PRIBILLA and PETTY *et al* conclude that the cholinesterase activity of separated plasma and erythrocytes can both be characterized by a mean value and its range.

The above mentioned routine determinations of cholinesterase activity were performed in the forensic chemical section of our institute by a modification of Michel's method (HUANG 1955), and the investigations were made on whole blood.

The object of our work was to obtain more comprehensive control material for the cholinesterase activity of autopsy blood determined by automatic titration, with a view to answering three questions 1) Is erythrocyte cholinesterase activity a better criterion than plasma cholinesterase activity for detecting a phosphostigmine poisoning? 2) What is the applicability of various *choline esters as substrates* in such examinations? 3) How important is the haemoglobin concentration for the evaluation of cholinesterase activity in whole blood from dead bodies?

The following *symbols* will be used

AChE erythrocyte cholinesterase, acetylcholinesterase.

BuChE plasma cholinesterase, butyrylcholinesterase.

ChE cholinesterase, not specified.

The symbols in brackets indicate the enzyme activity The following substrate designations are used ACh for acetylcholine, MeCh for methacholine (acetyl-β-methylcholine) and BuCh for butyrylcholine.

Material and Method

The material used for the investigation consisted of 165 blood samples. *The phosphostigmine group* included 15 blood samples from individuals who died after oral intake of phosphostigmines. *The control group* comprised 140 samples from autopsies, when the available information did not indicate the action of phosphostigmines. Finally 10 blood samples from *live individuals* were examined.

The control group includes 47 samples from hospital autopsies, and 93 from legal autopsies Out of the 93 individuals 16 had died from poisoning, 77 from other causes. For the hospital material we did not investigate the causes of death. Approximately 60 / of the control group samples were taken from men approximately 40% from women ages ranged from 2 to 96 years. Six samples from the legal autopsy group were taken from children under 15 years old most of the other samples were from individuals of the age group 40–65 years. Most of the hospital material came from old subjects, the youngest being 39 years old and 38 of the 47 individuals over 75 years old. The blood samples in the phosphostigmine group were taken from 2 females and 13 males of the age group 1–73 years. The samples from live individuals were taken from 2 women and 8 men in the age group 25–50 years.

The post-mortem blood samples came from routine autopsies those taken at legal autopsies were from the heart or the great veins those from hospital autopsies taken by syringe from the heart. *The blood samples from live subjects* were taken by venipuncture coagulation was prevented by heparin.

Haemolysates As a rule post-mortem samples were more or less haemolysed and often contained large and small clots. To facilitate pipetting, all enzyme determinations were made on *whole blood haemolysates* 1 + 4 The blood samples were mixed by turning them upside down slowly and mechanically for 5–10 minutes, major clots having been broken up in advance. Two ml samples of blood were rinsed into 8 ml water measured by a burette. Haemolysates and blood samples were kept in small plastic bottles at approximately −20°

Haemoglobin was determined in haemolysates and whole blood as cyanmethaemoglobin against Acuglobin Hemoglobin Standard (Ortho Pharmaceutical Corporation). In the determinations, 10 ml diluent and 20 µl blood or 100 µl haemolysate were used in order to obtain suitable extinctions. When examining samples of low haemoglobin concentration we used correspondingly larger volumes of blood and haemolysate, with suitable corrections in the calculations. Statistics and diagrams were based on the haemolysate determinations converted to terms of whole blood.

Apparatus. The cholinesterase activity was determined by automatic titration with a Radiometer titrator (TTT1a) connected to a titrigraph (SBR2), as described by JENSEN-HOLM, LAUSEN, MILTHERS & MOLLER (1959)

Reagents

Sodium hydroxide For the determinations 100 mM–NaOH were used, except for a few samples of 30 mM–NaOH The strength of the base was determined by potentiometric titration of a carbon dioxide-free potassium hydrogen tartrate solution.

Other reagents The substrates used were acetylcholine iodide and butyrylcholine iodide (Hoffmann-la Roche) as well as methacholine bromide (Ph. Dan. 1948 Addendum). From substrates and salts (sodium chloride and sodium chloride + magnesium chloride) were made solutions of a concentration 20 times greater than the concentrations given in Table 1 The substrate solutions were stored at 2 C.

Table 1

Reaction mixtures

The concentrations given *refer* to the reaction mixture after addition of the substrate solution. Whole blood is added in the form of 500 or 1000 µl of haemolysate (1 + 4). The last column shows the corrections for non-enzymatic substrate hydrolysis, which are to be subtracted from the total activities measured, expressed as µmol acid × min.$^{-1}$ × ml whole blood^{-1}

Substrate	Whole blood ml	Mineral salts: NaCl (mM)	Mineral salts: $MgCl_2$ (mM)	Non-enzymatic substrate hydrolysis
Erythrocyte cholinesterase				
4.0 mM acetylcholine	0.1	150	40	0.071
16.0 mM acetylcholine	0.1	150	40	0.285
12.5 mM methacholine	0.2	150	40	0.029
Plasma cholinesterase				
5.0 mM butyrylcholine	0.2	150	40	0.024
5.0 mM butyrylcholine	0.2	150	nil	0.022

Activity determination procedure

Distilled water 8.5 or 8.0 ml, was measured by burette, and 500 or 1000 µl haemolysate along with 500 µl salt solution were added. The mixture was heated to 38° while being aerated with carbon dioxide-free nitrogen. During the activity determination itself, the glass was covered with a loosely fitting lid, and only the space over the liquid was swept with nitrogen, to prevent contamination by the carbon dioxide of the air After reading the pH, and titrating to pH 7.40 we checked for possible spontaneous acid or base liberation. We then added 500 µl substrate solution and recorded the enzyme activity for 5–10 minutes. The recorded activities were calculated as micromoles of acid liberated per minute per millilitre of whole blood. The activities were corrected for non-enzymatic substrate hydrolysis by subtraction of the corrections given in the last column of Table 1 Five activity determinations were made for each haemolysate, three for AChE (substrates 4 and 16 mM ACh and 12.5 mM MeCh) and two for BuChE (substrate 5 mM BuCh), with magnesium ions present or absent. No duplicate determinations were made.

Non-enzymatic substrate hydrolysis. The corrections are relatively small, and they were therefore determined by the technique described below Protein-free systems were employed, the salt concentrations being as stated in Table 1 Fairly high concentrations of the substrates were added, 30–35 mM ACh, 100–140 mM MeCh and 35–70 mM BuCh. As titrant 7.6 mM-NaOH were used, and finally the sensitivity of the titrator was increased approximately fourteen times by a special device.

In these circumstances the hydrolysis per minute was

of acetylcholine	178	micromoles per mole present	
of methacholine	46.6	– – –	
of butyrylcholine	96.0	–	(Mg present)
of butyrylcholine	88.5	– – –	(Mg absent).

Material and Method

The material used for the investigation consisted of 165 blood samples. *The phospho-stigmine group* included 15 blood samples from individuals who died after oral intake of phosphostigmines. *The control group* comprised 140 samples from autopsies, when the available information did not indicate the action of phosphostigmines. Finally 10 blood samples from *live individuals* were examined.

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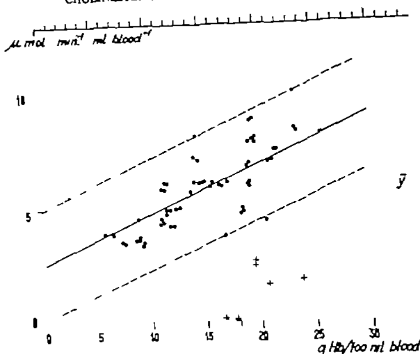


Fig. 2. Erythrocyte cholinesterase and haemoglobin concentration. Substrate 16 mM acetylcholine (B/Hb in table 3) (Notation from fig. 1 used)

adjust the pH of the mixture to 7 40. Spontaneous non-specific acid liberation, like the one formerly described by us in connection with experiments on tissue, was not observed during these examinations of blood. We did, however observe spontaneous non-specific base liberation (cf JENSEN-HOLM 1961), for after adjusting to pH 7 40 but before adding substrate, we sometimes found a slight rise in pH of the reaction mixture These rises were, however negligible compared with the pH fall after adding the substrate (titrator disconnected) they were therefore ignored.

The number of determinations (n) made in connection with the five series of analyses amounted to less than 140, as a few analyses were eliminated, either because the titration curves were irregular or because the samples showed an extremely high activity

Erythrocyte cholinesterase Figs. 1–3 show [AChE] determined with the substrates 4 and 16 mM ACh and MeCh, and it appears from these diagrams that there is a distinct regression between the esterase activity and the haemoglobin concentration. The numerical expressions of this conditional (two-dimensional) distribution are given in table 3 With a view to avoiding confusion, the symbols "s_{con}" and "s_{mar}" have been used

Results

The haemoglobin concentration showed an extremely large range, 1 8–28 2 g Hb/100 ml but the distribution within the range was found by probit analysis to be normal High haemoglobin concentrations occurred more frequently in legal autopsy samples than in hospital autopsy samples

The cholinesterase determinations When water haemolysate and salt solution had been mixed, the samples from live subjects showed a pH of 7 0–7.2, whereas the pH of the post mortem samples was lower generally 6 0–6 5 a quantity of 50–100 μmol base/ml blood was often required to

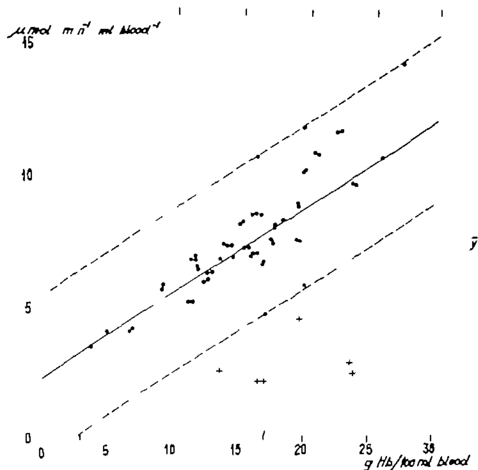


Fig. 1 Erythrocyte cholinesterase and haemoglobin concentration.
Substrate 4 mM acetylcholine (A/Hb in table 3)

The *abscissae* represent haemoglobin concentrations, the *ordinat s* enzyme activities. Values for the control group are shown by open circles, those for live subjects by solid circles and those for the phosphostigmine group by crosses.

The continuous oblique line is the regression line, and the broken, parallel lines indicate the 95 per cent limits of the conditional distribution (Y ± 1 96 s_{res}). To the right are shown the mean and the 95 per cent limits for the marginal distribution f the enzyme acti ities ($\bar{y}$ ± 1.96 s_{mar}).

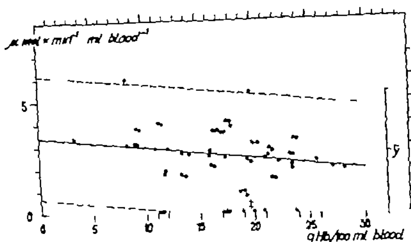


Fig. 4. Plasma cholinesterase and haemoglobin concentration.
Substrate 5 mM butyrylcholine, magnesium present (D/Hb in table 3)
(Notation from fig. 1 used)

zero in the hospital group of this analytical series. The variation in the enzyme activities alone, the marginal (one-dimensional) distribution, shows a normal distribution by probit analysis, and mean values as well as standard deviations are given in table 2. In figs. 1–3 the mean values as well as the 95 % limits are seen on the right.

Plasma cholinesterase Fig. 4 shows the results of determinations of [BuChE] with a magnesium containing reaction mixture the calculations are given in tables 2 and 3 The regression, being here negative, is not so pronounced as in the [AChE] determinations, but both regression- and correlation-coefficients differ significantly from zero at $P > 99$ % The regression is linear according to the criteria already laid down. [BuChE] was determined for each blood sample with magnesium present and with magnesium absent in the reaction mixture a comparison between the two sets of analyses is given by the regression equation *E/D* in table 3 The facts that $b = 1.028$ and does not significantly differ from 1 and that $a_0 = -0.024$ and does not significantly from zero seem to indicate that the presence of magnesium has no effect on the BuChE determination. The high correlation coefficient between these two sets of analyses is taken as an expression of a fairly slight spread in the true activity values.

The blood samples from *live subjects were examined by the procedure* used for the autopsy samples. The results have not been included in the calculations they are, however marked on the diagrams in figs. 1–4 from which it appears that samples from live subjects and post mortem samples show similar activity levels.

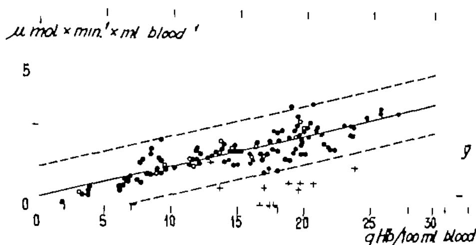


Fig. 3 Erythrocyte cholinesterase and haemoglobin concentration. Substrate 12.5 mM methacholine (C/Hb in table 3) (Notation from fig. 1 used)

in the present study for the standard deviations in the conditional distribution and the marginal distribution respectively The regression lines are marked on the diagrams as well as their 95 / probability limits. The hypothesis that the regressions should be linear is supported by the fact that the standardized deviations from the regression line $\frac{y - Y}{s_{con}}$ show normal distributions by probit analysis Both the regression and the correlation-coefficients differ significantly from zero by $P > 99.9\%$. The intersepts with the ordinate axis a_o, differ significantly from zero by $P > 99.9\%$ for the determinations with 4 and 16 mM ACh. The MeCh determinations are less reliable, and a_o does not differ significantly from

Table 2

Statistical analysis marg nal dist ib tio

The table shows the means and the standard deviations, s_{mar} of the haemoglobin concentrations and of the cholinesterase activities.
The haemoglobin concentrations are expressed as g Hb/100 ml whole blood The enzyme activities are expressed as µmol acid × min⁻¹ × ml whole blood⁻¹

	Mean	s_{mar}	n
Haemoglobin concentrations	15.4	6.20	138
Erythrocyte cholinesterase			
4 mM acetylcholine	7.29	2.60	138
16 mM acetylcholine	5.81	1.82	137
12.5 mM methacholine	2.10	0.89	136
Plasma cholinesterase			
5 mM butyrylcholine	2.60	1.34	129

cholinesterase, AChE, shows a maximal activity at approximately 2 mM ACh higher concentrations of ACh have an inhibitory effect on the enzyme. The plasma cholinesterase will show only slight activity at 2 mM ACh, and FLEISHER & POPE (1954), in measurements of normal whole blood, found that approximately 80 / of the activity measured was due to AChE. Our determinations of the erythrocyte cholinesterase with ACh as substrate were based on this principle.

In our investigations we employed 4 mM ACh and 12.5 mM MeCh, as they were found to be the concentrations that gave the highest activity in investigations of normal whole blood under the experimental circumstances described. A 16 mM ACh concentration is used in the above mentioned cholinesterase determinations conducted in the forensic chemical section. An arbitrary concentration of 5 mM BuCh was chosen.

Magnesium ions will activate AChE without affecting BuChE by making the determinations in a medium containing 150 mM sodium chloride and 40 mM magnesium chloride (AUGUSTINSSON 1950) a value of [AChE] can be obtained that constitutes a larger fraction of the total measured activity In our investigations magnesium had no effect on [BuChE].

The erythrocyte cholinesterase is bound to the erythrocytes on examining whole blood from normal live individuals, a linear regression can be found between [AChE] and the erythrocyte concentration, the latter expressed by the haematocrit value (AUGUSTINSSON 1955 SABINE 1959). It was not possible in our investigations to determine the haematocrit value, owing to haemolysis by using the haemoglobin concentration as reference, the linear regression was also found on examination of post mortem blood. In the determinations with 4 and 16 mM ACh and with MeCh almost equally high correlations were found between enzyme activity and haemoglobin concentration. An even higher degree of mutual correlation was seen in the determinations with 4 mM ACh and with MeCh (A/C in table 3). These phenomena, along with the fact that the enzyme activities found with 16 mM ACh were at a lower level than the determinations with 4 mM ACh (substrate inhibition) were assumed to indicate that AChE is the dominating enzyme in these three analytical series.

The intercept between the regression lines and the ordinate, a_0, is the enzyme activity at 0 g Hb/100 ml, and marks the theoretical mean value of the cholinesterase activity in pure plasma from the blood samples examined, determined with the corresponding substrate concentrations. In whole blood, [BuChE] is lower than a_0, owing to the presence of the erythrocytes. In figs. 1 and 2 this can be illustrated by drawing a line through the intercept between the regression line and the ordinate axis

Table 3

Statistical analysis conditional distribution

The table shows the equations for the regression lines, $Y = a_0 + bx$. For the coordinates given in the column y/x, the symbols used are A, analyses with 4 mM acetylcholine B with 16 mM acetylcholine C with 12.5 mM methacholine D with 5 mM butyrylcholine, magnesium present E with 5 mM butyrylcholine, magnesium absent. Hb denotes haemoglobin concentration Hb/Hb stands for haemoglobin in haemolysate/haemoglobin in whole blood s_{res} is the standard deviation for the variation about the regression line, *i.e* the deviation of an observed activity y from the corresponding value Y on the regression line s_{a_0} and s_b are the standard errors of the estimates a_0 and b of the regression line parameters r is the correlation coefficient.

The haemoglobin concentrations are expressed as g Hb/100 ml whole blood. The enzyme activities are expressed as µmol acid × min.$^{-1}$ × ml whole blood^{-1}

y/x	$Y = a_0 + bx$	n	s_{res}	s_{a_0}	s_b	r
A/Hb	Y = 2.21 + 0.33 x	138	1 61	0.37	0.022	0.79
B/Hb	Y = 2.24 + 0 22 x	137	1 25	0.29	0.017	0.73
C/Hb	Y = 0.28 + 0.12 x	136	0 53	0.12	0.007	0.81
D/Hb	Y = 3 36 − 0.049 x	129	1 31	0.31	0.019	−0.23
A/C	Y = 2.14 + 2.46 x	134	1.32	0.29	0.129	0.86
E/D	Y = −0 024 + 1 028 x	113	0.39	0 080	0.027	0.96
Hb/Hb	Y = *0.20 + 1 012 x*	*164*	*0.99*	*0.22*	*0.013*	*0.99*

Tests of significance

$t = r\sqrt{n-2}/\sqrt{1-r^2}$ $t = (a_0 - 0)/s_{a_0}$ $t = (b - 0)/s_b$ $t = (b - 1)/s_b$.

As was to be expected, *the phosphostigmine group* showed low activities, and the fact that the group departs distinctly from the control group will be discussed below The activities measured which have been marked on the diagrams, were not statistically examined. Some of the blood samples were not subjected to all five analyses

Discussion

Post mortem blood is often somewhat haemolysed and the present investigations were therefore made solely on whole blood As the two cholinesterases will both split acetylcholine, selective substrates are often used in whole blood analyses, such as methacholine for erythrocyte cholinesterase and butyrylcholine for plasma cholinesterase (AUGUSTINSSON 1955) the substrates mentioned were used in three of our analytical series. It is, however possible even with acetylcholine as substrate to obtain a practicable standard for the erythrocyte cholinesterase activity of whole blood for this purpose advantage is taken of the fact that the activities of the two enzymes differ in their dependence on substrate concentration Where as the plasma cholinesterase, BuChE, shows increasing activity with rising substrate concentration, the erythrocyte

plasma proteins in samples with a high content of haemoglobin such determinations were therefore not made.

Owing to the fact that results are widely dispersed, we did not – for this analytical series – investigate the possible influence of age, sex, state of health before death and so on, although it appears from the literature that these conditions will to a greater or less extent affect the [BuChE] in human subjects. However PRIBILLA (1957) did not, in investigations of plasma from post-mortem blood, find any clear effect of such factor

Our investigations have revealed a pronounced difference between the blood samples from legal autopsies and those from hospital autopsies. The regression line for blood from the hospital group alone showed a slope corresponding to that found for whole blood from live subjects. The regression line for blood from the legal autopsy group, however showed a lower slope, and this group had also a higher a_o-value than had the hospital group.

The legal autopsy group was thus found to be on a higher level than the hospital group, both in [BuChE] and in haemoglobin concentration it seems therefore as if these specimens showed a haemoconcentration. (The variation in haemoglobin concentrations found in the hospital group may be partly due to the sampling technique) It appears, from the available information about the time intervals between death and autopsy that approximately ⅘ of the hospital autopsies were made within 24 hours after death, whereas only approximately half of the legal autopsies were made by this time. It seems justifiable to assume that the haemoconcentration mentioned was caused by slow post-mortem liquid shifts, so slow that in our investigations they did not show themselves in samples taken from the body within 24 hours of death.

To sum up the result of this analytical series, it can be said that the spread, and particularly the frequency of extremely low enzyme activities was so pronounced that our [BuChE] determinations cannot serve as suitable control material.

Stability of the enzymes Certain of the legal autopsy blood samples were stored untreated for some time at 2° before being frozen. A graphic picture, showing the standardized deviations from the regression lines against the storing time, confirmed the widely held view that these enzymes are stable, for no clear tendency to fall can be detected. For example, after being stored for approximately 60 days at 2° five samples showed [AChE] and [BuChE] values located on or above the regression lines.

Non phosphostigmine poisonings

The legal autopsy group consisted of 16 deaths caused by poisoning with carbon monoxide, or with various drugs the poisoning was verified

with a slope corresponding to that of the regression line in fig. 4 The vertical distance between the abscissa axis and the regression line represents the average total activity for a given haemoglobin concentration, and this distance is divided into two parts by the new line, the lower part corresponding to [BuChE] the upper part, between the regression line and the new line corresponding to [AChE] For the concentration 15 g Hb/100 ml it was found that in the 4 mM ACh determinations [AChE] constituted approximately ¾ of the total activity and in the 16 mM ACh determinations, [AChE] constituted approximately ⅘ of the total activity Some reservations should be made about these figures, especially as a was determined by extrapolation, but they are in conformity with the findings of FLEISHER & POPE, 1/- that [AChE] made up approximately 80 / of the total activity in measurements with 2 mM ACh.

We did not investigate whether in our material [AChE] might be affected by age, sex, state of health before death, and so on. PETTY *et al* did not demonstrate any clear effect of these factors. Reports in the literature about live, both healthy and sick, individuals show that [AChE] is independent of sex and age and only slightly affected by illness (RIDER *et al* 1955 SHANOR *et al* 1961) However a fall or rise in erythropoiesis will cause a corresponding change in the [AChE] (SABINE 1959) We did, however investigate whether or not blood samples, taken at legal autopsies, differed from those taken at hospital autopsies it was found that in all three analytical series the two sub-groups were almost identical with the control group *in toto*

To sum up all three sets of [AChE] determinations gave an expression applicable to the erythrocyte cholinesterase activity of whole blood, but the 4 mM ACh determinations are preferable Application of 16 mM ACh will involve a fairly high correction for non-enzymatic substrate hydrolysis moreover at this concentration substrate inhibition is noticeable Methacholine is split by AChE only at a slow rate use of this substrate will therefore cause the total activity level to be fairly low which may prove inconvenient.

Plasma cholinesterase These determinations differ distinctly from the other analytical series in showing a negative regression of enzyme activity on the haemoglobin concentration as well as by the frequent occurrence of low values The negative regression is due to the fact that a rising haemoglobin concentration (cell volume) will entail a fall in the plasma content of whole blood To judge by the low value of the correlation coefficient, the haemoglobin concentration would not be the proper reference in this case the concentration of plasma proteins, especially the cholinesterase-containing fraction would presumably be a better reference. There seemed to be no suitable technique available for determining

the diagnosis of phosphostigmine poisoning. It is, however feasible by demonstrating a cholinesterase activity within the range of the control group to exclude with reasonable certainty the possibility of a phosphostigmine poisoning.

It must therefore be concluded that, in forensic chemical investigations, a combination of routine determinations of the erythrocyte cholinesterase activity in whole blood and haemoglobin determinations will constitute a valuable means of revealing, or more frequently of excluding, evidence of phosphostigmine poisoning.

Summary

The cholinesterase activity in autopsy blood was examined in a material including a *phosphostigmine group* (15 samples from legal autopsies after poisoning with parathion, etc.) and a *control group* (140 samples from legal autopsies and hospital autopsies).

The haemoglobin concentrations varied from 1.8 to 28.2 g Hb/100 ml.

The cholinesterase activity was determined in whole blood by automatic titration. The erythrocyte cholinesterase activity was determined with acetylcholine (4 mM and 16 mM) and methacholine (12.5 mM) as substrates. The control group showed a high correlation with the haemoglobin concentration and a positive linear regression. The plasma cholinesterase activity was determined with butyrylcholine (5 mM) as substrate. The control group showed a low correlation with the haemoglobin concentration and a negative linear regression it included many very low values.

Whole blood samples from live subjects did not differ from those of the control group.

The phosphostigmine group showed low erythrocyte cholinesterase activities with much overlap of the marginal distribution in the control group. If however the haemoglobin concentration is included in the evaluation, the two groups differ distinctly The plasma cholinesterase activities did not differ from those of the control group.

It is concluded that, in *forensic chemical investigations, determinations* both of erythrocyte cholinesterase activity in whole blood and of haemoglobin will prove a valuable selective, but not a specific, means of revealing or excluding a diagnosis of phosphostigmine poisoning.

Acknowledgements

This study was supported by a grant from Reinholdt W Jorck og Hustru s Fond.

Most of the enzyme determinations were made with the greatest care

by quantitative forensic chemical examinations By comparing the standardized deviations from the regression lines in our material, we found that neither [AChE] nor [BuChE] differed from those of persons dying from other causes of death. Any cholinesterase-inhibiting qualities that these poisons might possess must therefore presumably be reversible, so that probable inhibitions are repressed under the analytical conditions employed, namely a 50- or 100-fold dilution of the blood and the use of substrate concentrations that must be considered high in this connection (JENSEN HOLM 1961 JÓHANNESSON & LAUSEN 1961 WINTERINGHAM & DISNEY 1963)

Phosphostigmine poisonings

The material investigated included blood samples from 15 cases of poisoning with phosphostigmines, mostly parathion the irreversible cholinesterase inhibitions caused by these substances manifested themselves as low activities. A primary inspection of the [AChE] conditions will show as is clear from figs. 1–3 that there is a considerable overlap between the activities measured in the phosphostigmine group and the marginal distribution of [AChE] in the control group (the 95 % limits are seen to the right) But it appears that, when the haemoglobin concentration is included in the calculations, the conditional distribution shows 95 % limits that are much narrower and *there is a pronounced difference between the phosphostigmine group and the control group* In examinations of whole blood with such greatly varying haemoglobin concentrations as those found in our material, a mean value with its range, as used by PETTY *et al* (1958) can therefore not be considered suitable basis for comparison. It deserves notice, however that in examinations of samples from live subjects the variation in the haemoglobin concentration is so slight as to be negligible in this connection. Even in the conditional distribution, the phosphostigmine group is not completely distinct from the control group fig. 1 shows a single value from the control group among the phosphostigmine group values, and fig. 3 shows a single value from the phosphostigmine group among the control group values. A determination of [AChE] in whole blood combined with a haemoglobin determination may therefore be considered a selective, but not a specific, means of recognizing phosphostigmine poisoning. A [BuChE] determination in the available form seems, however to be less valuable, owing to the frequent occurrence of extremely low values in the control group A fundamental principle in forensic chemical analysis, *vl* that the cause of a poisoning should be established by more than one examination, also applies here a demonstration of a greatly reduced cholinesterase activity in whole blood is therefore not in itself sufficient to establish with certainty

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From the Research Division, AB Pharmacia, Uppsala, Sweden

Estimation of Vasodilator Drug Effects in Mice by Measurements of Paw Skin Temperature

By

Wolfgang Richter

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The measurement of skin temperature is well established clinically as an indicator of peripheral blood flow especially in the diagnosis of peripheral occlusive vascular disease. A survey of the methodology is to be found in a recent review by BURTON (1948) and by HARDY (1961) of the physiology of temperature regulation.

Skin temperature measurements in various species, in both conscious and anaesthetized animals, have been performed for studying thermoregulation and peripheral circulation. Among the species used were rats (WINTER & FLATAKER 1953 HEROUX 1959), rabbits (CRISMON & FUHRMAN 1947), cats (PERKINS & MAO CHIH LI 1947 STRÖM 1950A KUNDT BRUCK & HENSEL 1957) dogs (HEMINGWAY & LILLEHEI 1950 STRÖM 1950B VAN DER GHINST 1956) and monkeys (DELGADO, FULTON & LIVINGSTON 1947). However skin temperature measurements in conscious animals for the study of vasodilator drug effects seem not to have had widespread use.

The method presented here, involving conscious mice, allows the detection of drugs that increase peripheral blood flow by measuring paw temperatures before and after drug administration. It appears to fulfil the requirements for a simple and rapid method of studying peripheral vasodilation induced by drugs. Since the method has given satisfactory results during several years use, details of the technique and examples of its application will be described.

Materials and Method

Animals. Albino mice of either sex, weighing 18–30 g, from the NMRI strain, are used.

Apparatus. A miniature thermistor of the thermometer type, whose resistance decreases rapidly with increase of temperature is employed. The temperature-sensitive

and interest by Mr Henry Bleg and Mr K A. Jensen both chemical students at the time the author is most grateful for their valuable help.

The author thanks Dr Jørgen Voigt, the Department of Forensic Medicine and Dr Hemming Poulsen then head of the pathological department, Sundby Hospital as well as their staffs for collecting the blood specimens

Apparatus and substrates were kindly placed at our disposal by a grant from Nordisk Insulinfond and Statens almindelige Videnskabsfond.

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Fig. 2. Photograph showing position of mouse during temperature measurements of the palmar skin of the fore paw. The thermistor may be applied in a similar way to the plantar skin of the hind paw.

contact with the skin. Temperature equilibrium is attained within 30 sec after placing the thermistor against the palm, and the maximum temperature is then read from the galvanometer.

Methodological Investigations. The reproducibility of consecutive measurements was tested by determining fore-paw temperature in ten mice twice at an interval of 30 seconds.

Normal paw skin temperatures were determined in 200 mice of both sexes, and their frequency distribution was plotted.

A possible difference between sexes was studied by comparing normal paw skin temperatures in 100 males and 100 females.

The effect of saline injection and handling on paw temperature was studied in both the quantitative and the screening version of the method, in order to establish the upper limits of normal paw skin temperature. In the quantitative method paw temperature was measured in 30 groups of four mice each, measurements being made before, and 15, 30, 45 and 60 minutes after injection of 0.20 ml of 0.9% saline. In the screening procedure, paw skin temperature was measured in 50 single mice before, and 30 minutes after saline injection.

The possibility of a difference between fore- and hind-paw skin temperatures was studied by measurements on 40 male mice.

The comparative rise in skin temperatures of the fore- and hind-paws was studied before and 15, 30, 45 and 60 minutes after s.c. injection of various doses of acepromazine into mice of four groups.

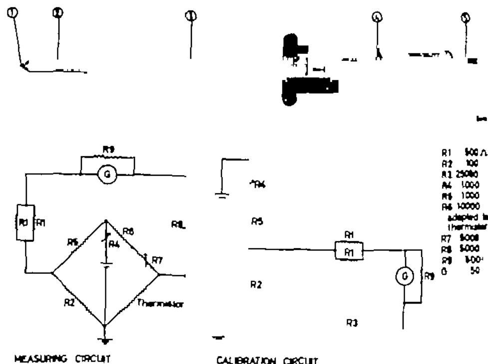


Fig. 1

Top Thermistor unit
1) Temperature sensitive thermistor tip
2) Glass tube containing thermistor and connections
3) Protective metal tube
4) Rubber cuff serving as handle
5) Connecting cable to Wheatstone bridge, 100 cm length

Bottom Wheatstone bridge connected to thermistor
General view of the electrical circuits.

spherical tip has a diameter of 0.75 mm. The thermistor is shown in fig. 1 and is available from Philips Ltd (Type B 8 320 05 P/2K2.) A more recent type may also be used (E 205 CE/P 2K2S)

A Wheatstone bridge and a galvanometer are used for measuring the change in resistance of the thermistor The electrical circuit is shown in fig. 1 The bridge is calibrated for measurements between 22–42° Within this range, the temperature can be read directly from the galvanometer with an accuracy of 0 1 The range of the instrument does not allow determinations of temperature below 22°)

Most experiments have been performed at room temperatures from 18–22°

Procedure for temperature measurements The photograph in fig. 2 shows a mouse during measurement of the palmar skin temperature of the fore paw The animal is seized by the back, a sufficiently large skin fold being taken between the thumb and index finger the little finger keeping the tail fixed. In this position the animal keeps its fore limbs extended and stretched away from the body The thermistor is placed from below against the palm of one fore paw This leads to elicitation of the grip reflex, so that the claws close tightly around the tip of the thermistor facilitating good

) The bridge and thermistor unit is manufactured on demand by M Leif S ahn, Pharmacia AB, Uppsala.

FREQUENCY DISTRIBUTION OF THE PALMAR SKIN TEMPERATURES OF THE FORE PAW IN MICE

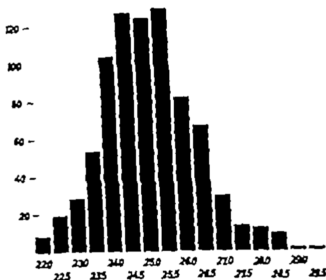


Fig. 3. Distribution around the mean of the skin temperatures of the fore paw of 800 mice of both sexes at room temperatures from 18–22°
Ordinates are number of mice, abscissae are skin temperatures of the fore paw in °C.
Mean and standard deviation 25.0 ± 1.3
0.88 % of all measurements fall outside the range of ± 2.6 from the mean.
The measuring range of the instrument used did not allow temperature determinations below 22

the slightly biphasic top of the fore-paw temperature histogram shown in fig. 3

Experimental results for the effect of saline injection and handling on fore-paw temperature in the quantitative method are shown in table 1 It is seen that a slight, but not significant, rise due to injection and handling occurs. When estimating drug effects, therefore, the lower limits for a significant rise of skin temperature in any group of four mice at 15 30, 45 and 60 minutes after injection are 1 9 1.4 2.3 and 2.1 respectively ($p = 0.05$).

In the screen procedure, the results for the effect of a saline injection and handling on fore-paw skin temperature of 50 mice were in 30 mice a temperature elevation, in 14 a temperature depression, and in 6 no change within 30 minutes of the injection. The average result was an increase of

In some exploratory experiments the effect of ephedrine in decreasing paw temperature was studied in guinea pigs (see discussion)

Quantitative method. For detailed dose-response and duration studies, paw temperature measurements were made on mice in groups of four before and 15 30, 45 and 60 minutes after injection of various doses of the compound to be tested. The mean value from four animals was calculated Rectal temperature was measured simultaneously For duration studies, measurements were continued until the skin temperature had returned to normal values. Increases in skin temperature by at least 2.0° and decreases in rectal temperature by at least 2.0° were considered as significant effects at a level of P = 0.05

Screening method. The mouse paw temperature method has been made part of a standard observation procedure along with determination of toxicity (RICHTER & CAMPBELL 1962). Consecutive groups of three mice are injected i. p. with the test compound at doses on the fixed dose scale (mg/kg) 1600 800, 400 200 etc. Skin temperature is measured in each animal once before and 30 minutes after injection. An animal showing a rise of the fore paw temperature of at least 2.0° is considered a positive reactor

Any dose level producing at least two positive reactors is considered to induce a significant rise in skin temperature.

In this way the presence or absence of increase in skin temperature and the dose range producing such effect may be rapidly determined for any compound. The lowest dose producing a significant rise in skin temperature is designated the minimal effective dose.

In a similar way rectal temperature is measured in each animal before and 30 minutes after drug injection Each animal with a decrease in rectal temperature of at least 2.0° is considered to show a significantly lowered body temperature. Any dose producing at least two positive reactors is considered to produce significantly reduced body temperature. The simultaneous determination of skin temperature and body temperature facilitates the classification of observed drug effects.

Results

Normal skin temperatures of the fore paw and effects of some factors on it

Two consecutive series of measurements obtained on 10 mice gave values of 25 3 ± 1 0 and 25 2 ± 1 0 (m. ± s.) For any single animal the difference between the first and second measurements did not exceed 0 3

The distribution of fore paw skin temperature values of 800 mice of both sexes, collected over two years of testing, is shown in fig 3 It is seen that values are distributed around a mean of 25 0 with a standard deviation of 1 3 Of all measurements, 0 88 / fall outside the range of ±2.6 s. from m

Comparison of fore-paw skin temperatures in samples of hundred male and female mice each gave the values 24 6 ± 1 2 and 24 1 ± 0 9 The difference is not statistically significant. It may however account for

Skin temperature of fore and hind paws and their comparative rise induced by a vasodilator drug

Skin-temperatures of the fore and hind paw were determined in 40 mice. No significant difference could be found, the mean values and their standard deviations amounting to 24.7 ± 1.8 and $24.5 \pm 2.0°$ respectively.

After administration of vasodilator drugs, it was observed that the temperature of the fore paw always rose more than that of the hind paw. Results with acepromazine in table 2 illustrate these findings. The difference is more pronounced after large doses.

Dose response studies on drugs by the quantitative method.

Dose response curves are shown in Fig. 4 for the skin temperature increasing, i. e. vasodilator effect of low s. c. doses of acepromazine, reserpine and tolazoline. The experiments demonstrate that acepromazine reserpine and tolazoline at such levels produced significant vasodilation without a significant decrease in rectal temperature. Results with acepromazine appear of special interest, because they show that vasodilation can be demonstrated at doses lower than those producing depression in rectal temperature. This means that vasodilation is a more specific sign than rectal temperature depression in characterising acepromazine and similar phenothiazines.

The effect of large doses of acepromazine and tolazoline on paw skin-temperature and rectal temperature is shown in fig. 5. Results with acepromazine show that vasodilation could be observed in spite of a marked decrease in rectal temperature. It is also evident that even large s. c. doses of tolazoline depressed rectal temperature only moderately. The vasodilating effect of these two compounds was maximal at 20–80 minutes after s. c. injection and lasted for about 160 minutes after injection.

Effect of drug administration on fore paw temperature in the "screening" procedure

To determine the discriminating power of the present test procedure, this was applied to a series of 64 clinically established drugs, including typical representatives of the more commonly employed drug categories. The results are given in table 3. A significant *increase* in paw skin temperature has been taken as criterion of vasodilator activity (see Discussion).

In 22 compounds of various drug groups, vasodilator activity varying in relative strength, was found. Most of these drugs are sympathicotropic compounds, known to interfere with catecholamines or their receptors.

Table 1

Influence of a single saline injection and of repeated handling on fore paw temperature in 30 groups of four mice each.

	Minutes after a single i. p. injection of 0.20 ml of 0.9 ⁄ saline				
	0	15	30	45	60
Paw skin temp C m ± s.	24 6 ± 0 7	24 9 ± 0 8	4 8 ± 0.6	25 1 ± 0.9	25 1±0 8
Mean increase above initial value of paw temperature in C	–	0 3	0.2	0.5	0.5
Lower limit for signif icant rise in skin temp as C above initial paw temper ature (p = 0 05)	–	1 9	1 4	2.3	2.1

0 3 with a standard deviation of ±1 0 For any single mouse, a reduc tion of 1 7 or an increase of 2.3 is considered as a significant change in paw temperature (p = 0 05) For routine purposes a rise in fore-paw temperature of at least 2.0 was considered to indicate a significantly increased blood flow

The significant decrease in paw temperature obtained in guinea-pigs after ephedrine injection is shown in table 4 (see also under Discussion).

Table 2

Comparative skin temperature rise of fore- and hind paw after different doses of acepromazine.
Measurements were made in 1– groups of four mice each per dose level before, and 15, 30, 45 and 60 minutes after injectio of test compound. Maximum skin temperature rise usually occurred from 15–30 minutes after s. c. injection.

Experiment no.	Acepromazine dose in mg/kg s.c.	Maximum rise in sk n temperature in C		Excess fore paw temp. rise in C
		Fore paw	Hind paw	
1	0 02	1 4	1 1	+0.3
2	0.10	4.3	2.6	+1 7
3	0.10	3 3	1 7	+1 6
4	0.25	4 1	..0	+2.1
5	0.25	8 8	6.4	+2.4
6	0.50	7 5	4 5	+3 0
7	1 00	6.0	1 7	+4 3

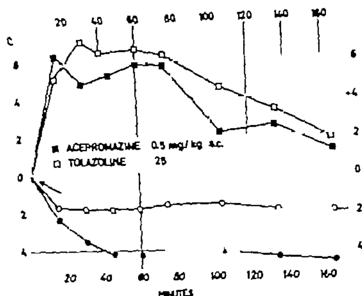


Fig. 5

Squares fore paw temperature. Circles rectal temperature.
Abscissa time in minutes after injection. Ordinate mean deviation from pretreatment temperature in C.
Determinations of the skin temperature of the fore paw and of rectal temperature were made after single large doses of acepromazine and tolazoline. One group of four mice each was used per dose level. Room temperature 20 C. Significant increase in paw temperature at p 0.05 $+2.0°C$ or more. Significant decrease in rectal temperature at p 0.05 -1.9 C or more. The time of injection is indicated by an arrow

They include reserpine, sympathicolytics, psychoactive phenothiazines and sympathomimetic drugs. Among those with vasodilator activity but not clearly classified as sympathicotropic, are compounds belonging to various drug groups apomorphine, aminophylline, trimethadione, caffeine, hyoscine, atropine, pethidine, imipramine, promethazine, diphenhydramine and tetracaine. Among the remaining 43 compounds, none exhibited vasodilator activity even at the highest dose levels tolerated. Some of these latter compounds are sympathicotropic drugs, for example, ephedrine and yohimbine.

Discussion

Any rise in skin temperature may be caused either by increased body temperature or increased local blood flow or both. Therefore, the need for simultaneous measurements of rectal temperature is evident. In mice

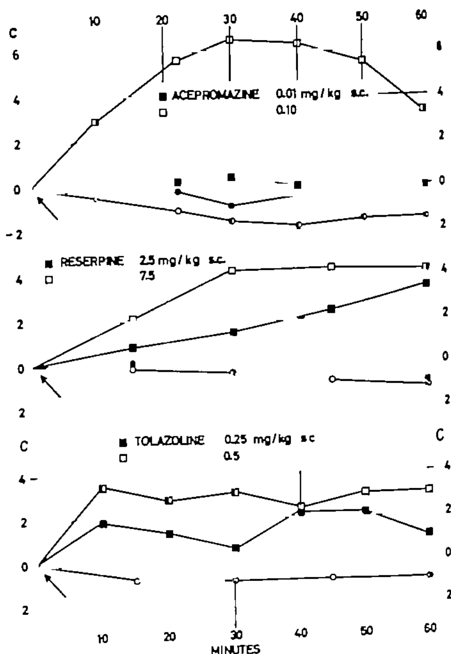


Fig. 4

Squares fore paw temperature. – Circles rectal temperature.
Abscissa time in minutes after injection. Ordinate mean deviation from pretreatment temperature in C.
Determinations of the skin temperature of the fore paw and of rectal temperature after single near-threshold doses of acepromazine reserpine, and tolazoline. One group of four mice each was used per dose level. Room temperature 21 22
Significant increase in paw temperature at p 0.05 +2.0 or more.
Significant decrease in rectal temperature at p 0.05 −1 9 or more.
The time of drug injection is indicated by an arrow

(Table 3)

1 Compound	2 Drug Category	Vasodilating action, minimal effective dose, expressed as	
		mg/kg	per cent of i. p. LD50
Tripelennamine	–	–	
Gallamine	Muscle relaxant		
d-Tubocurarine	–	–	
Papaverine	Plain muscle depressant	–	–
Diphenyl-dimethyl-aminobutane.			–
Dibucaine	Local anaesthetic	–	–
Dimethisoquine			–
Lidocaine			
Procaine			
Pramoxine			
Pentetrazol	Convulsive agent		
Strychnine			
Histamine	Varia		–
Serotonin			

Barbiturates were tested as sodium salts.
Column 4 the i. p. LD50 values (24 h observation time) were estimated by injecting groups of three mice each with doses of the scale (mg/kg) 1.5, 3, 6, 12, 25, 50, 100, 200 400, 800, 1600.

kept at environmental temperatures of 18–22° only few drugs produce a significant rise in body temperature. In the course of a systematic investigation of 65 drugs belonging to various types of clinically used drugs (RICHTER & CAMPBELL 1962) only three were found to possess a strong calorigenic effect in mice and to increase body temperature significantly they were isoprenaline, amphetamine and strychnine. The strong calorigenic action of isoprenaline has been reported previously (MOORE & UNDERWOOD 1960) and that of amphetamine is also well known.

Except for these drug categories, all others producing an increase in skin temperature do so by increasing local blood flow In resting animals at normal room temperatures, increased local blood flow is generally due to vasodilation.

The quantitative relationships between skin temperature changes and blood flow are complicated (HARDY 1961). This is, however of little importance in relation to the method described here, since increased skin temperature is here only an indicator of increased blood flow Clinically used vasodilators produce a marked rise in skin temperature extending over a varying range of dose levels. Similar properties in new compounds may thus be detected by the present procedure.

The determination of decrease in skin temperature as an indicator of

Table 3

Peripheral vasodilating properties detected by the mouse paw temperature test on various clinically used drugs.

1	2	Vasodilating action, minimal effective dose, expressed as	
Compound	Drug Category	mg/kg	per cent of i.p. LD50
Acepromazine	Tranquilizer	0.06	0.1
Isoxsuprin	Sympatholytic	0.8	0.5
Tolazoline	–	0.8	1.0
Noradrenaline	Sympathomimetic	1.5	8.0
Reserpine	Tranquilizer	3.0	2.0
Chlorpromazine	–	3.0	2.0
Naphazoline	Sympathomimetic	3.0	4.0
l-adrenaline	–	3.0	67.0
Phenoxybenzamine	Sympatholytic	6.0	8.0
Azapetine	Vasodilator	25.0	16.0
Apomorphine	Emetic	25.0	16.0
Promethazine	Tranquilizer	25.0	67.0
Tetracaine	Local anaesthetic	25.0	67.0
Imipramine	Antidepressive agent	50.0	67.0
Diphenhydramine	Antihistaminic	50.0	67.0
Isoprenaline	Sympathomimetic	100.0	16.0
Aminophylline	Plain muscle depressant	100.0	33.0
Pethidine	Analgesic	100.0	67.0
Caffeine	CNS stimulant	200.0	67.0
Atropine sulphate	Parasympatholytic	200.0	67.0
Hyoscine HBr	–	400.0	67.0
Trimethadione	Anticonvulsive	800.0	33.0
Amphetamine	CNS stimulant	–	
Cocaine	–		
Pipradol	–	–	
Pentymal	CNS depressant		
Meprobamate	–	–	
Neallymalum	–	–	
Pentobarbital	–		–
Phenobarbital	–	–	
Thiomebumal	–	–	
Hydroxidion	–	–	
Carbacholin	Parasympathomimetic		
Neostigmine	–	–	
Benactycine	Parasympatholytic	–	–
Hyoscine methonitrate			
Diphemanil	–	–	–
Ephedrine	Sympathomimetic	–	
Yohimbine	Sympatholytic	–	
Acetylsalicylic acid	Antiinflammatoric analgesic	–	–
Amidopyrine		–	
Phenylbutazone	–	–	–
Codeine	Analgesic		
Morphine		–	
Iproniazid	Antidepressive agent		
Klordiazepoxide	Tranquilizer		
Diphenylhydantoin	Anticonvulsive	–	–
Phensuximide	–		
Antazoline	Histamin antagonist	–	–
Chlorpheniramine	–		

(*Table 3*)

1 Compound	2 Drug Category	Vasodilating action, minimal effective dose, expressed as mg/kg	 per cent of i. p. LD50
[illegible]			–
Gallamine	Muscle relaxant		
d-Tubocurarine			
Papaverine	Plain muscle depressant		
Diphenyl-dimethyl-aminobutane.			
Dibucaine	Local anaesthetic		
Dimethisoquine			
Lidocaine			
Procaine			
Pramoxine			
Pentetrazol	Convulsive gent		
Strychnine			
Histamine	Varia		
Serotonin			

Barbiturates were tested as sodium salts.
Column 4 the p. *LD50* values (24 h observation time) were estimated by injecting gro ps of three mice each with doses of the scale (mg/kg) 1 5 3 6, 12, 25, 50 100, 200 400, 800, 1600.

kept at environmental temperatures of 18–22° only few drugs produce a significant rise in body temperature. In the course of a systematic investigation of 65 drugs belonging to various types of clinically used drugs (RICHTER & CAMPBELL 1962) only three were found to possess a strong calorigenic effect in mice and to increase body temperature significantly they were isoprenaline, amphetamine and strychnine. The strong calorigenic action of isoprenaline has been reported previously (MOORE & UNDERWOOD 1960) and that of amphetamine is also well known.

Except for these drug categories, all others producing an increase in skin temperature do so by increasing local blood flow In resting animals at normal room temperatures, increased local blood flow is generally due to vasodilation.

The quantitative relationships between skin temperature changes and blood flow are complicated (HARDY 1961) This is, however of little importance in relation to the method described here, since increased skin temperature is here only an indicator of increased blood flow Clinically used vasodilators produce a marked rise in skin temperature extending over a varying range of dose levels. Similar properties in new compounds may thus be detected by the present procedure.

The determination of decrease in skin temperature as an indicator of

Table 4

Vasoconstrictor effect of i p ephedrine on guinea pigs detected by decrease in paw skin temperature.

Male guinea pigs, weighing 300–500 g, are used. To facilitate measurements of paw temperature, an assistant holds the animal immobilized with fore limbs extended. Figures in the table are mean deviations from initial temperature in C. In 11 groups of 4 animals each, the normal paw skin temperature at a room temperature of 23 was found to be 33.9 ± 0 8 In any treated group a temperature decrease of 1 7 or more was considered as indicating a vasoconstrictor drug effect.

Number of animals	Time in min after i. p injection	5	10	15	20
4	Saline group	+0 6	±0	+0.7	+0.9
4	Ephedrine group (Dose 5 mg/kg)	−2.3	−2.4	−4 6	−3.0

vasoconstrictor action in mice is more difficult, because of the low paw skin temperature, 25 0 of this species at normal room temperatures. Animals that normally have a high paw skin temperature would therefore be more suitable for measuring vasoconstrictor drug effects. Exploratory experiments on the guinea pig, which normally has a paw skin temperature of 33–35 confirmed this view The results in table 4 indicate that a marked decrease in skin temperature of guinea pigs is obtained within 15 minutes after ephedrine injection.

The psychoactive phenothiazines and the adrenolytic vasodilators, tolazoline and isoxsuprin show a high degree of specificity by the paw skin temperature method described here For chlorpromazine and acepromazine this is emphasized by the fact that they produce a skin temperature increase at low doses that do not depress body temperature, reduce motor activity or induce ptosis (RICHTER & CAMPBELL 1962)

Azapetine and isoxsuprin also induce skin temperature increase at low doses.

Among the easily measurable signs in mice, the rise in skin temperature seems to be surpassed in specificity only by the mydriatic effect of some anticholinergics

Both sympathicolytic and sympathicomimetic drugs induced significant rises in skin temperature exemplifying the complexity of vascular pharmacology

The method described makes it possible to detect both strongly and weakly active vasodilators. Besides the drugs used clinically with a well-known weak vasodilator action, such as aminophylline and caffeine, weak vasodilator action has also been found in some compounds conventionally not classified as vasodilators such as apomorphine and trimethadione.

Summary

A simple, rapid and sensitive method for detecting and measuring quantitatively vasodilator drug effects in conscious mice by determination of paw skin temperature is described. The normal paw skin temperature was determined in 800 mice and the effects of various factors on it were studied. Out of 64 clinically used reference drugs of various categories, 22 produced a significant increase in paw skin temperature. The ten most active compounds included 4 adrenolytics, 2 sympathomimetics, 3 major tranquilizers and apomorphine.

The powerful peripheral adrenolytic action of the psychoactive phenothiazines, acepromazine and chlorpromazine, could be demonstrated at doses lower than those producing reduced motor activity depression of body temperature or ptosis.

Acknowledgements

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Experimental Disturbance of Porphyrin Metabolism and of Liver Catalase Activity in Guinea Pigs and Rabbits

By

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(Received March 7 1964)

The finding of SCHMID & SCHWARTZ (1952) that hepatic catalase activity is decreased in rats and rabbits poisoned with apronal NFN (= sedormid ®) led to investigations into the activity of this haemoprotein in other types of porphyria. Though hexachlorobenzene(C_6Cl_6) proved to have no demonstrable effect on catalase activity in rats (SCHMID 1960), diethyl 1 4 dihydro-2,4 6 trimethylpyridine 3,5 dicarboxylate (DDTD) was found substantially to suppress the activity of the catalase in rats (DE MATTEIS & PRIOR 1962) and in rabbits (HAEGER ARONSEN 1962 b).

The present supplementary investigation of the effect of C_6Cl_6 on the catalase activity in guinea pigs and rabbits was prompted by the observation of inter-species differences in susceptibility to this compound (DE MATTEIS *et al* 1961)

Judging from the literature about the effect of lead on various steps in the synthesis of haem in the erythroblast (for review see HAEGER ARONSEN 1960), it is not known whether the synthesis of liver catalase is affected by lead. It was therefore considered useful to study the activity of this enzyme in lead poisoned animals.

The "haematoporphyrinuria" described by STOKVIS (1895) in sulphonal-poisoned rabbits could be explained by an increased excretion of copro-porphyrin (WALDENSTRÖM & WENDT 1939). We have studied the excretion of porphyrins and their precursors, as well as the catalase activity in the liver of sulphonal-poisoned rabbits.

It is known that 3-amino-1,2,4-triazole (AT) suppresses catalase activity in rats (HEIM *et al* 1955 & 1956), as well as that of δ-aminolaevulic acid dehydrase in mice (TSCHUDY & COLLINS 1957) It was therefore thought worth while to study AT for any effect on porphyrin metabolism.

Ström, G Effect of hypothalamic cooling on cutaneous blood flow in the unanesthetized dog. *Acta Physiol Scand.* 1950 21 271 277 (B)
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Each of 10 rabbits (Nos. 11–20) was given a single subcutaneous injection of 4 % lead acetate in water (w/v) in the back. The dose was 125 mg of lead acetate/kg b.w.

Five rabbits (Nos. 21–25) were given sulphonal in doses of 0.4–1.8 g/kg b.w. The substance was given as a 2 % water solution by stomach tube. The dosage scheme is shown in fig. 3.

Five rabbits (Nos. 26–30) received a daily dose of 100 mg of 3-amino-1,2,4-triazole (Light & Co.) in 1 % aqueous solution. The dose was administered subcutaneously in the back.

The 24-hour outputs of urinary δ-aminolaevulic acid (ALA) *), porphobilinogen (PBG), uroporphyrin (UP) and coproporphyrin (CP) and of faecal CP and protoporphyrin (PP) in guinea pigs Nos. 9–17 were determined twice before the first injection of C_6Cl_6 and the day after each dose of the compound. Corresponding determinations were also made in all the rabbits after administration of C_6Cl_6, sulphonal or 3-amino-1,2,4-triazole (AT). The excretion of PBG and UP in the urine was, however, not determined in the animals that received lead acetate. The animals were placed in metabolism cages to permit separate collection of urine and faeces.

All the intoxicated animals (guinea pigs Nos. 9–17 and rabbits Nos. 1–30) were killed the day after the last dose of the test substance, and the livers were removed for measurement of the catalase activity. The concentration of "green porphyrins" in the liver was determined in rabbits Nos. 21–30.

Immediately after removal, the livers from guinea pigs and rabbits were washed and homogenized, as described by Dounce & Shanewise (1950). The mean wet weight per millilitre was 0.98 g (0.93–1.02) and the corresponding dry weight, 0.081 g (0.054–0.108). For estimation of the catalase activity each homogenate from the guinea pigs was diluted with distilled water to 0.2 %, 0.1 % and 0.05 %, and each homogenate from the rabbits was diluted to 4 %, 2 % and 0.4 %.

The catalase activity was determined by the method of Feinstein (1949). Each sample was analysed twice; the mean values are given. The activity was expressed as millimoles of $NaBO_3$ decomposed by 0.5 ml of homogenate under the conditions of the method used. The calculations were made after correcting the wet weights of the original homogenates to 1.00 g per ml.

The other methods used have been previously described (Haeger 1958, Haeger-Aronsen 1960, 1961, 1962 a, b).

Results

Guinea pigs

The catalase activity found in the livers of 8 apparently healthy guinea pigs and of 9 animals that had been given C_6Cl_6 are given in figs. 4 A and B, respectively. The number of millimoles of $NaBO_3$ decomposed by 0.5 ml of the 0.2 % homogenates ranged between 0.52–0.29 and 0.46–0.20 in the two groups.

The values found for the urinary δ-aminolaevulic acid (ALA), porphobilinogen (PBG), uroporphyrin (UP) and coproporphyrin (CP) and

*) Abbreviations used: AT 3-amino-1,2,4-triazole, ALA δ-aminolaevulic acid, PBG: porphobilinogen, UP uroporphyrin, CP coproporphyrin, PP: protoporphyrin.

1*

Material and Methods

Seventeen guinea pigs (11 males, 6 females), weighing between 0.4 and 0.8 kg, and 30 rabbits (14 males, 16 females), weighing between 2.6 and 3.8 kg, were used.

The catalase activities of the livers of 8 apparently healthy guinea pigs (Nos. 1–8) were estimated. Nine guinea pigs (Nos. 9–17) were given hexachlorobenzene (Schuchardt, München) in doses of 0.4–0.9 g/kg body weight (b.w.). The substance was suspended in 15–20 ml of water and given by stomach tube. The dosage scheme is shown in fig. 1.

Ten rabbits (Nos. 1–10) were given hexachlorobenzene (C_6Cl_6) in doses of 1.4–2.8 g/kg b.w. The substance was suspended in 15–20 ml of water and given by stomach tube. The dosage scheme is shown in fig. 2.

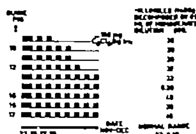


Fig. 1. Doses of hexachlorobenzene (C_6Cl_6) given to 9 guinea pigs.

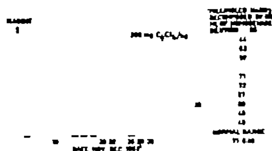


Fig. 2. Doses of hexachlorobenzene (C_6Cl_6) given to 10 rabbits.

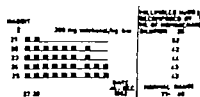


Fig. 3. Doses of sulphonal given to 5 rabbits.

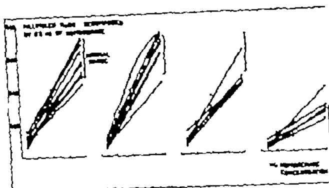


Fig. 6. $NaBO_3$ decomposition related to concentration of liver homogenate.
A. 10 hexachlorobenzene-intoxicated rabbits.
B. 10 lead-intoxicated rabbits.
C. 5 sulphonal-intoxicated rabbits.
D 5 aminotriazole-intoxicated rabbits.

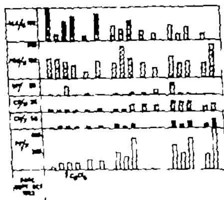


Fig. 7 Mean urinary 24-hour excretion of δ-aminolaevulic acid (ALA), porphobilinogen (PBG), uroporphyrin (UP) and coproporphyrin (CP) as well as of faecal CP and protoporphyrin (PP) by 10 hexachlorobenzene (C_6Cl_6)-intoxicated rabbits. Arrow indicates first day of treatment with C_6Cl_6.

The concentration of "green porphyrins" in the liver was normal in the two groups studied (C and D).

Figs. 7 9 and 10 give the mean values found for the urinary excretions of ALA, PBG UP and CP and for the faecal excretions of CP and PP in groups A, C and D the mean urinary ALA and CP and faecal CP and PP in group B are given in fig. 8

In group A the excretions of UP and CP in the urine and of CP in the faeces were slightly increased. The faecal PP was substantially increased. The urinary levels of ALA and of CP were raised in group B.

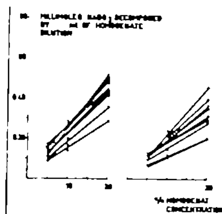


Fig. 4 $NaBO_3$ decomposition related to concentration of liver homogenate.
A. 8 normal guinea pigs.
B. 9 hexachlorobenzene intoxicated guinea pigs.

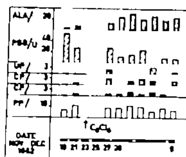


Fig. 5. Mean urinary 24-hour excretion of δ-aminolaevulic acid (ALA), porphobilinogen (PBG) uroporphyrin (UP) and coproporphyrin (CP) as well as of faecal CP and protoporphyrin (PP) by 9 hexachlorobenzene (C_6Cl_6)-intoxicated guinea pigs. Arrow indicates first day of treatment with C_6Cl_6.

faecal coproporphyrin (CP) and protoporphyrin (PP) are given in fig. 5.

All of the components studied were excreted in normal amounts except ALA, which was slightly higher

After 3–4 days treatment with C_6Cl_6 the animals appeared to be tired. After a few more days they showed a coarse tremor of the limbs. All the animals lost weight during the experiment.

Rabbits

The values found for the catalase activity of the livers of 10 animals given C_6Cl_6, 10 given lead, 5 given sulphonal and 5 given AT are plotted in figs. 6 A B C and D It is clear that the catalase activity was largely normal (0 71–0 48 millimoles $NaBO_3$ decomposed by 0 5 ml of 4% homogenate) in the first two groups, slightly depressed in 4 of 5 rabbits in group C and substantially decreased in all 5 animals in group D

The amount of CP excreted in the urine in group C was somehwat large. In this group the faecal *PP* rose greatly in all the animals on the first day after administering sulphonal, but then returned to normal. The other metabolites measured in these three groups and in group D were excreted in roughly normal amounts.

After 2 weeks treatment with C_6Cl_6, 3 of the animals (Nos. 1 2 and 7) showed neurological symptoms in the form of coarse tremor and paresis of the hind legs. After 1 week s treatment all the animals that received C_6Cl_6 appeared weak and had a poor appetite, oliguria and constipation.

Discussion

Table 1 shows the excretion of porphyrins and their precursors, as well as the concentration of PP in the erythrocytes and the activity of liver catalase, in patients with porphyria acuta intermittens (PAI) and in different species of animals treated with apronal, diethyl 1 4-dihydro-2,4,6-trimethylpyridine-3,5-dicarboxylate, griseofulvin, hexachlorobenzene, lead, sulphonal or 3-amino-1,2,4-triazole.

Administration of the first five compounds, which are not chemically related, leads to a considerable disorder of porphyrin metabolism. In the rat and the rabbit, treatment with apronal, DDTD griseofulvin sulphonal or AT leads to a more or less substantial suppression of catalase activity in the liver Administration of C_6Cl_6 to the guinea pig or rabbit is, however not followed by any change in hepatic catalase activity

In none of the types of experimental porphyria hitherto produced do the symptoms and biochemical pattern coincide with what is seen in hereditary acute intermittent porphyria in man. The forms of porphyria most closely resembling human porphyria are those produced by apronal and DDTD But in these experimental porphyrias the catalase activity is reduced, which does not occur in human acute porphyria (GRAY 1950). Neither do these compounds produce the types of neurological disorders seen in PAI That treatment with apronal produces peripheral paralysis in rats with pyridoxine or pantothenic acid deficiency (DE MATTEIS 1962) is noteworthy especially since these two vitamins are necessary for the normal biosynthesis of haem (SCHULMAN & RICHERT 1956).

The clinical and biochemical responses to C_6Cl_6 differ widely among species. Man responds with development of photosensibility hepatomegaly and porphyrinuria, but not with abdominal or neurological symptoms (SCHMID 1960 CETINGIL & ÖZEN 1960), whereas the laboratory animal species hitherto tested react with neurological symptoms, particularly coarse tremor of the limbs and occasionally convulsions (OCKNER



Fig. 8. Mean urinary 24-hour excretion of δ-aminolaevulic acid (ALA) and coproporphyrin (CP) as well as of faecal CP and protoporphyrin (PP) by 5 lead (Pb)-intoxicated rabbits. Arrow indicates first day of treatment with lead.

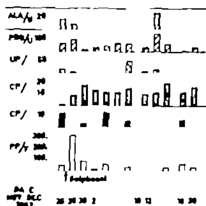


Fig. 9 Mean urinary 24-hour excretion of δ-aminolaevulic acid (ALA) porphobilinogen (PBG) uroporphyrin (UP) and coproporphyrin (CP) as well as of faecal CP and protoporphyrin (PP) by 5 sulphonal-intoxicated rabbits. Arrow indicates first day of treatment with sulphonal.

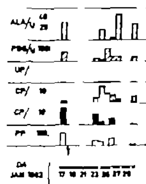


Fig. 10. Mean urinary 24-hour excretion of δ-aminolaevulic acid (ALA) porphobilinogen (PBG), uroporphyrin (UP) and coproporphyrin (CP) as well as of faecal CP and protoporphyrin (PP) by 5 aminotriazole (AT)- intoxicated rabbits. Arrow indicates first day of treatment with AT

concentration in rabbits and rats is abnormally elevated.

The only two types of provoked disorders in porphyrin metabolism with an increased concentration of protoporphyrin in the red blood cells are those produced by administration of griseofulvin (DE MATTEIS & RIMINGTON 1963) or lead (VIGLIANI & WALDENSTRÖM 1937). The concentration of PP in the red blood cells is also increased in the two known types of hereditary erythropoietic porphyria, namely congenital porphyria and erythropoietic protoporphyria.

In the other types of experimental porphyria the metabolic disorders are probably localized mainly in the liver (SCHMID & SCHWARTZ 1952 GOLDBERG 1955 RICHARDS 1960 SCHMID 1960) In DDTD intoxicated guinea pigs, the activity of ALA synthetase increases in the mitochondria of the liver cells, but other enzymes involved in porphyrin biosynthesis retain their normal activity (GRANICK & URATA 1963) The rate of synthesis of porphyrins in these cells is normally dependent on the activity of ALA synthetase. It is therefore only natural that any increase in activity of this enzyme should be accompanied by an increase in the rate of porphyrin synthesis.

The suppression of catalase activity in apronal intoxication can be traced back to the inhibited synthesis of this enzyme (SCHMID & SCHWARTZ 1952 & 1955 SCHMID *et al.* 1953 SCHMID *et al.* 1955). Whether any causal relationship exists between the low catalase activity in certain types of experimental porphyria and co-existing disturbances of porphyrin metabolism is still obscure.

As expected from previous investigations (WALDENSTRÖM & WENDT 1939), the sulphonal-poisoned rabbits showed a slightly increased urinary excretion of CP and a normal urinary excretion of porphobilinogen. The moderately increased excretion of CP and the slightly decreased catalase activity in 4 of the 5 experimental animals were probably due to liver damage by the drug. Sulphonal by itself thus produces porphyrinuria but not porphyria. Latent acute intermittent porphyria can, however become manifest after ingestion of sulphonal, and this is what probably occurred in the patients described by STOKVIS (1889) and SALKOWSKI (1891).

Investigation into the effect of aminotriazole on porphyrin metabolism in rabbits revealed that all of the metabolites studied were excreted in normal amounts. The inhibitory effect of AT on catalase activity in the liver was clear in our material.

Summary

In guinea pigs and rabbits, hepatic catalase activity was not affected by administration of hexachlorobenzene (C_6Cl_6). Nor was the activity in rabbits affected by lead. The C_6Cl_6-intoxicated guinea pigs excreted a

Table 1

Concentration of δ-aminolaevulic acid (ALA) porphobilinogen (PBG), uroporphyrin (UP) and copreporphyrin (CP) in urine, of CP and *protoporphyrin (PP) in faeces and of PP in* red blood cells and hepatic catalase activity in patients with acute intermittent porphyria as well as in animals with different types of experimental porphyria.

Porphyrinmetabolism deranged by	Urine ALA	Urine PBG	Urine UP	Urine CP	Faeces CP	Faeces PP	RBC PP	Liver catalase activity
Porphyria acuta intermittens (PAI)	++	+++	+++)	++	+	+	N	N
Apronal Rabbits, rats	+	+++	++)	+	+	++	N	–
Diethyl 1 4-dihydro 2,4 6-trimethylpyridine *3,5-dicarboxylate* (DDTD 2,4 6)								
Rabbits	+	+++	+++)	+++	+	+++	N	–
Rats	++	+++	+++)	++	?	?	?	–
Griseofulvin Rats	++	++	(+)	+	++	+++	+++	–
Hexachlorobenzene (C_6Cl_6)								
Homo	N	(+)	++	++	N	N	N	?
Rabbits, rats	+)	+)	++	++	+	++	N	N
Guinea pigs, mice	(+)	N	N	+	N	N	N	N
Lead								
Homo	+++	N	(+)	++	N	N	+++	?
Rabbits, rats, Guinea pigs	+++	+	+	++	N	N	+++	N
Sulphonal Rabbits	N	N	N	(+)	N	N	?	(–)
3-amino-1 2,4-triazole (AT) Rabbits	N	N	N	N	N	N	?	–

(+) to +++ = Increased. N = Normal (–) to – = Decreased
? = Information not available.
) Chiefly made *in vitro* from PBG
) Terminal rise in the rats.

& SCHMID 1961) but only slight cutaneous reactions. Though the urinary excretion of porphyrin precursors is largely normal in man guinea pigs, mice and rabbits, it may be *terminally increased in rats* (DE MATTEIS *et al* 1961) In contrast to other species studied the faecal porphyrin

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slightly increased amount of δ-aminolaevulic acid (ALA) in the urine, whereas the rabbits excreted porphyrins in increased amounts in both urine and the faeces The rabbits treated with lead showed a metabolic reaction, with markedly increased urinary excretions of ALA and coproporphyrin.

In the rabbits, administration of sulphonal or 3-amino-1,2,4-triazole led to a slight or substantial depression respectively in liver catalase activity Moderate coproporphyrinuria was observed in the sulphonal-poisoned animals, but all of the porphyrins and their precursors studied were excreted in normal concentrations by the rabbits that received triazole.

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slightly increased amount of δ-aminolaevulic acid (ALA) in the urine, whereas the rabbits excreted porphyrins in increased amounts in both urine and the faeces. The rabbits treated with lead showed a metabolic reaction, with markedly increased urinary excretions of ALA and coproporphyrin.

In the rabbits administration of sulphonal or 3-amino-1,2,4-triazole led to a slight or substantial depression respectively in liver catalase activity Moderate coproporphyrinuria was observed in the sulphonal-poisoned animals, but all of the porphyrins and their precursors studied were excreted in normal concentrations by the rabbits that received triazole

Acknowledgements

Thanks are accorded to Mrs Mariann Nilsson and Miss Kristina Broman for skilful technical assistance The investigation was supported by grants from the Assurance Company Folksam Stockholm, Sweden.

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From the Department of Medicine Karolinska Sjukhuset, Stockholm
(Professor Henrik Lagerlöf M D)

Effect of a Quaternary Phenothiazine Derivative (Aprobit®) on Gastric Secretion

By

Åke Hanngren

(Received March 5 1964)

It has long been known that quaternary ammonium compounds have both ganglion blocking and anticholinergic properties (LONGINO *et al* 1950 ABBOT *et al* 1952) Quaternary substances of the phenothiazine group such as 10-(α-dimethylaminopropionyl)-phenothiazine methobromide (secergan ®) possess similar properties (DAHLBOM *et al* 1953 WIEDLING 1958) These substances have been used to treat disturbances of gastrointestinal motility and secretion (KIRSNER *et al* 1953 KIRSNER & PALMER 1953 TOMENIUS 1957)

In 1960 CARLSSON *et al* described a histamine antagonist that can be chemically characterized as a quaternary oxyethyl derivative of promethazine-1-(10-phenothiazinylmethyl)-ethyl 2 hydroxy-ethyldimethylammonium chloride (aprobit ®) Since this phenothiazine derivative has no sedative action it is suitable for day-time medication Of 110 patients who received aprobit ® for allergic manifestations, one reacted with nausea and vomitting. Otherwise no side-effects were observed Xerostomia and mydriasis did not occur

In animal experiments aprobit ® was found to have an antiacetylcholine action, which also included some ganglion blocking action along with a brief antiadrenergic effect (ALBANUS *et al* 1961) These pharmacological observations prompted me to study the effects of aprobit ® on gastric secretion in human subjects

Method and Material

The apparent basal gastric secretion (LEVIN *et al* 1951) was determined for one hour in persons who had fasted for twelve hours. Each subject was tested with and without administration of aprobit ® the interval between these tests being one week. Aprobit

® was given intravenously in a dose of 0.1 mg per kg of body weight. During the test the subject reclined in a chair and a duodenal tube (Mediplast no. 16, Levin) was passed down to the pyloric antrum. Swallowing of saliva was prevented by a saliva aspirator. The fasting gastric secretion was *aspirated for 15 minutes with a syringe*. Aprobit ® was then injected, and the duodenal tube was coupled to a continuous water-suction system at a negative pressure of 45 mm Hg for 60 minutes. The aspirated gastric secretion was collected after three periods, each of 20 minutes, and its volume and acidity (colorimetric determination of pH) were measured.

Ten healthy persons (five males and five females) were tested. None of them had any history of gastric symptoms. Their ages ranged from 21 to 27 years.

Supplementary experiments were made on ten other healthy persons (nine males and one female) in the same age group. The method was the same, except that aprobit ® was given by mouth, 50 mg three times on the day before the test and 50 mg two hours before the test. Each person in this series underwent two control tests, one before and one after the aprobit test.

In two more persons the effect of intravenously administered aprobit ® on gastric secretion was analyzed after stimulation with histamine (0.01 mg/kg body weight by subcutaneous injection).

Statistical analysis of the results was by Student's t-test.

Results

Immediately after intravenous injection of aprobit ® all the test subjects showed a transient increase in pulse rate (fig. 1). The blood pressure was not affected, and no xerostomia or mydriasis was observed.

The total volume of basal gastric secretion during one hour in the first series of ten persons was 543 ml. After intravenous injection of aprobit ®

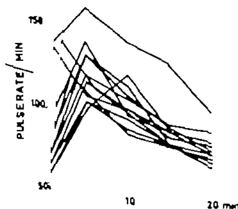


Fig. 1. Pulse rate after intravenous injection of aprobit ® (0.1 mg/kg body weight)

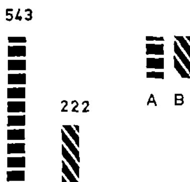


Fig. 2. Total gastric secretion (ml) during 1 hour from 10 persons without (A) and after (B) intravenous injection of aprobit ®.

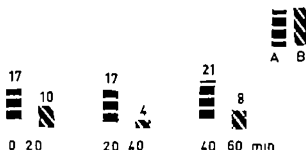


Fig. 3 Mean volume of gastric secretion in 10 persons during 20-minute periods without (A) and after (B) intravenous injection of aprobit ®

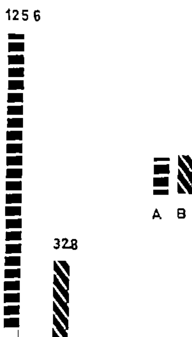


Fig. 4. Mean of total HCl (mEq/litre) during 1 hour in 10 persons. A = control tests B = after intravenous injection of aprobit ®.

the corresponding volume in the same persons was 222 ml (fig. 2). The secretion invariably diminished after administration of aprobit ®. The fall was greatest in the 20 to 40 minute period, when the total volume was 43 ml, compared with 166 ml when aprobit ® had not been given (fig. 3).

When aprobit ® had been given by mouth, no statistically significant difference was found between the two control tests and the aprobit test in total volume of gastric secretion: the respective values were 743, 497 and 493 ml. Five of the ten test subjects, however, showed diminished secretion after ingestion of aprobit ®.

The effect of aprobit ® on production of hydrochloric acid is illustrated in fig. 4–6. Both the total HCl production during one hour (fig. 4) and the peak value in any 20 minute period (fig. 5, table 1) showed great reduction after intravenous injection of aprobit ®. This reduction was particularly marked in the 20 to 40 minute fraction (fig. 6), for which the figure 2 mEq per litre applied to only one of the test subjects and the remainder had achlorhydria.

When aprobit ® had been given by mouth, the values for total HCl showed a trend in the same direction, but the differences were not statisti-

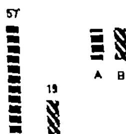


Fig. 5. Mean of peak HCl (mEq/litre) values in 10 persons. A = control tests. B = after intravenous injection of aprobit ®.

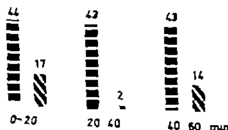


Fig. 6. Mean HCl (mEq/litre) in 10 persons during 20-minute periods. A = control tests. B = after intravenous injection of aprobit ®.

Table 1

Apparent basal gastric secretion and free HCl after intravenous injection of aprobit ®

Gastric secretion (ml)

	Total during 1 hour			0–20 minutes			20–40 minutes			40–60 minutes		
	ml	t =	significance	ml	t =	significance	ml	t =	significance	ml	t =	significance
Control tests	54 3 ± 7 6	5 97	xxx	16.8 ± 5 7	2.57	x	16.6 ± 6 8	5.22	xxx	20.9 ± 9 7	3.57	xx
After aprobit ®	22.2 ± 5 7			9 9 ± 2.0			4.3 ± 3.2			8 0 ± 6 1		

Free HCl (mEq/litre)

	Maximum during 1 hour			0–20 minutes			20–40 minutes			40–60 minutes		
	mEq	t =	significance	mEq	t =	significance	mEq	t =	significance	mEq	t =	significance
Control tests	56 7 ± 35 6	.82	x	44.3 ± 35 7	2.14	x	42.8 ± 38.1	3.53	xx	43 4 ± 38 0	2.18	x
After aprobit ®	19.2 ± 1.2			16.8 ± 19 6			2.0			14 0 ± 19 8		

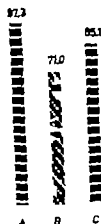


Fig. 7 Mean of total HCl (mEq/litre) during 1 hour in 10 persons.
A & C = control tests.
B = after oral administration of aprobit ®.



Fig. 8 Mean total acidity (mEq/litre) in 10 persons during 20-minute periods.
A = control tests B = after intravenous injection of aprobit ®.

cally significant (fig. 7). However achlorhydria was found in five of the ten test fractions from the 20 to 40 minute period and in three from the 40 to 60 minute period.

The total gastric acidity showed the same trends as the values for free HCl (fig. 8)

Table 1 presents statistical findings for the volume of gastric secretion and the amount of free HCl in the experiments with intravenously administered aprobit ®

Two persons received histamine in customary dose in order to stimulate gastric secretion aprobit ® did not affect their secretion.

Discussion

Quantitative evaluation of gastric secretion requires a method that prevents escape of secretion *via* the pylorus as well as admixture of duodenal secretion and swallowed saliva (IHRE 1938) These requirements can be met with the Lagerlöf Ågren double-lumen tube plus a saliva aspirator But the procedure then becomes unnecessarily time-consuming for pilot tests in which the object is only to demonstrate possible antisecretory effect of drugs One must keep in mind however that when a single lumen tube is used it is not the true, but the apparent, gastric secretion that is measured This can nevertheless be adequate when the conditions are identical in all tests and only one relationship is to be determined.

Histamine-induced gastric secretion is not subject to the same fluctuations as the basal secretion on different test occasions in the same individual (IHRE 1938) But in healthy persons below the age of 30 years the basal secretion during one hour in the morning is parallel with the secretion in response to test meals or histamine stimulation (LEVIN *et al* 1951) Individual fluctuations of basal secretion from time to time were discussed by KIRSNER & PALMER (1953) although there is no precise arithmetical correlation satisfactory reproducible results for the individual subject may be obtained under identical conditions of study

With these considerations in mind the results in table I can be assessed Intravenously administered aprobit ® greatly reduced the total volume of gastric secretion Maximal reduction was found 20 to 40 minutes after the injection when the difference from control values was highly significant. Free hydrochloric acid likewise was significantly less after intravenous injection of aprobit ® with the greatest fall in the 20 to 40 minute period, when nine of the ten test subjects showed achlorhydria. The effect on gastric secretion was transient which could be attributed to the intravenous route of administration and the low dose of aprobit ®

The rapid initiation and short-duration of the effect on gastric secretion and the absence of side-effects in these experiments suggest that clinical trials are advisable for investigating the spasmolytic effect of aprobit ® for instance in roentgenologic studies of the biliary tract

Orally administered aprobit ® produced no statistically significant depression of gastric secretion from that of controls, although a tendency to reduction in acidity was observed. Quaternary ammonium compounds are known to be slowly absorbed from the digestive canal probably because of their high ionization at the prevalent pH (LEVINE, BLAIR & CLARK 1955) In animal experiments aprobit ® was less well absorbed than promethazine (HANSSON & SCHMITERLÖW 1961), and this can explain the weaker response to oral compared with intravenous administration of

aprobit ®. In my experiments aprobit ® was given for one day only. It is not possible at present to judge whether or not longer medication or higher dosage would influence gastric secretion sufficiently to make aprobit ® clinically useful in, for instance, duodenal ulcer. Adequately prolonged antiacidity without side-effects has not yet been obtained with other substances of similar type (KIRSNER & PALMER 1953).

None of the quaternary ammonium compounds in doses free from side effects produces significant depression of *histamine-induced* gastric secretion. This I found to be true also of aprobit ®. The striking atropine-like effect of aprobit ® on gastric secretion appears before any possible effects on salivation or pupils become discernible. Xerostomia and mydriasis were *not found* in the present experiments. The pulse rate, however, showed a transient rise. The transient fall in blood pressure reported from animal experiments (ALBANUS *et al.* 1961) did not occur in the human subjects in my experiments.

Summary

The inhibitory effects on basal gastric secretion of the quaternary compound 1-(10-phenothiazinylmethyl)-ethyl-2 hydroxy-ethyldimethyl ammonium chloride (aprobit ®) was investigated in human subjects. Intravenous injection of aprobit ® was followed by decrease in the volume and acidity of the secretion. This effect was transient but statistically significant. Nine of ten subjects, none of whom had any history of gastric symptoms, showed achlorhydria 20 to 40 minutes after the injection. Oral administration of aprobit ® did not significantly alter the volume or acidity of the gastric secretion.

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Names approved by The Nordic Pharmacopoeia Council[1] (NFN Names)

(Received March 6, 1964)

The Nordic Pharmacopoeia Council announces its approval of NFN[2]) names for the substances in the list below. These names may be used should the substances be included in the forthcoming Nordic Pharmacopoeia, in one or more of the Nordic national pharmacopoeias and official formularies or in addenda to any of these.

The substances for which these names have been approved are identified by giving their chemical names first, secondly the most frequently used non-proprietary names, including the International Non-Proprietary Names, approved by WHO, and thirdly a selection of the best known registered trade names and other names of pharmaceutical specialities (preceded by the letter ®) applying either to the substance itself or to salts of it or to preparations of which it is the active principle. This information is given in a similar manner to that in the Council's publication "NFN-navne"[3]) both in volume of information and in use of abbreviations[4]).

Names approved in 1963[5])

NFN-name	*Other Names*
Acetohexamidum (BAN, pINN, USAN)	1-(4-Acetylphenylsulfonyl)-3-cyclohexylcarbamide. ® Dimelor, Dymelor (Antidiabetic)
Acidum dibunicum sodium salt: natrii dibunas (BAN, pINN)	2,6-Di-tert. butylnaphthalenesulfonic acid-(1)[6]). BAN: Sodium Dibunate, pINN: Natrii dibunas (sodium salt). ® Adaxin, Becantex, Becantyl. (Antitussic)
Acidum foscolicum (pINN)	P,P'-Bis(1-carboxy-1-hydroxyethyl)phosphinic acid = 2,2'-phosphinicodilactic acid.

1) The General Secretary: Fack, Stockholm 60, Sweden.
2) NFN: Abbreviation of the Nordic name ("Nordiska Farmakopé-nämnden") for the Nordic Pharmacopoeia Council.
3) NFN-navne, 2nd edition, Copenhagen, Helsingfors, Reykjavik, Oslo and Stockholm, with Addendum 1961.
4) The abbreviation "USAN" indicates, that the name is approved by the American Medical Association – United States Pharmacopeia Nomenclature Committee.
5) Names approved in 1962, see this journal vol. 20: 113–114, 1963.
6) The chemical constitution is not yet quite elucidated.

NFN name	*Other Names*
Acidum tyropanoicum sodium salt natrii tyropanoas (pINN USAN)	2-(3 Butyramido-2,4 6-tri-iodobenzyl)butyric acid. pINN Natrii tyropanoas, USAN Sodium Tyropanoate (sodium salt) ® Bilopaque. (X ray contrast medium)
Aconiazidum (pINN)	(2 Isonicotinoylhydrazonomethyl)phenoxyacetic acid. ® Phenoxalid. (Chemotherapeutic)
Aethyli cartrizoas (pINN)	(3 5-Diacetamido-2,4 6-tri-iodobenzoyloxy)acetic acid ethyl ester pINN Ethylis cartrizoas. (X ray contrast medium)
Aethyli dibunas (BAN pINN USAN)	3 6-Di-tert butylnaphthalenesulfonic acid-(1) ethyl ester°) BAN USAN Ethyl Dibunate. pINN Ethylis dibunas. (Antitussive)
Amfecloralum (pINN USAN)	2-(2,2,2 Trichlorethylidenamino)-1 phenylpropane. USAN Amphecloral. ® Acutran (Anorexigenic)
Amfomycinum (BAN DCF pINN)	Antibiotic, produced by Streptomyces canus. BAN Amphomycin DCF Amfomycine.
Angiotensinamidum (BAN DCF pINN USAN)	N [1 [N [N [N-(N-(N^2-Asparaginylarginyl)valyl)-tyrosyl]valyl]histidyl]propyl]-3-phenylalanine = valy-hypertension II-asp-β-amide. BAN USAN Angiotensin Amide. DCF Angiotensinamide. R Hypertensin-Ciba Hypertensin N (Hypertensive)
Aprofenum (pINN)	,2 D phenylpropionic acid (2-diethylaminoethyl)ester (Spasmolytic)
Atropini oxidum (pINN USAN)	Atropine-N-oxide. pINN Atropini oxydum. USAN Atropine Oxide Hydrochloride (chloride) ® Xtro. (Spasmolytic)
Azatepum (pINN USAN)	P P Bis(aziridinyl-(1))-N-ethyl-N-(1 3 4-thiadiazolyl-(2))phosphinamide Azatepa. USAN Azatepa. (Antineoplastic)
Azathioprinum (pINN USAN)	6-(1 Methyl-4-nitroimidazolyl-(5)-thio)purine. (Antineoplastic)
Bamipin m (BAN pINN)	4-(N Benzylanilino)-1 methylpiperidine. ® So entol. (Antiallergic)
Bendazolum (pINN)	2 Benzylbenzimidazole. (Spasmolytic)
Benzindopyrinum (pINN USAN)	1 Benzyl 3-(2 pyridyl-(4)-ethyl)indole. USAN Benzindopyrine Hydrochloride (chloride). (Psycho-sedati e)

NFN-name	*Other Names*
Benzopyrronium bromide, benzopyrronii bromidum (pINN)	3-Benziloyloxy-1,1-dimethylpyrrolidiniumhydroxide. pINN Benzopyrronii bromidum, USAN Benzopyrronium Bromide (bromide). (Anticholinergic)
Benzpiperylonum (pINN)	4-Benzyl-1-(1-methylpiperidyl-(4))-3-phenyl-3-pyrazolinone-(5). Ⓡ Reublonil. (Antirheumatic)
Bibenzonium bromide, bibenzonii bromidum (pINN)	(2-(1,2-Diphenylethoxy)ethyl)trimethylammoniumhydroxide. pINN Bibenzonii bromidum (bromide). Ⓡ Lysobex, Modipectol, Sedobex. (Antitussive)
Bromchlorenonum (pINN,USAN)	6-Bromo-5-chloro-2-benzoxazolinone. Ⓡ Vinyzene. (Fungicide, antibacterial)
Brometenaminum (DCF pINN)	Equimolecular complex of bromoform and methenamine. DCF Brométhénamine. (Hypnotic)
Bromindionum (pINN,USAN)	2-(4-Bromophenyl)indandione-(1,3). Ⓡ Halinone. (Anticoagulant)
Broxaldinum (pINN)	Benzoic acid (5,7-dibromo-2-methyl)quinolyl-(8) ester (Antibacterial)
Broxichinolinum (pINN)	5,7-Dibromo-8-hydroxyquinoline. pINN Broxyquinolinum. (Antibacterial)
Bucetinum (BAN,pINN)	N-(3-Hydroxybutyryl)-4-phenetidine. (Analgesic)
Butamoxanum (pINN)	2-(Butylaminomethyl)-1,4-benzodioxane. (Psycho-sedative)
Butynaminum (pINN)	N-Tert-butyl-N,1,1-trimethyl-2-propynylamine. (Antihypertensive)
Camylofinum (pINN)	N-(2-Diethylaminoethyl)-2-phenylglycine isopentyl ester Ⓡ Avacan, Ltcosia. (Spasmolytic)
Capreomycinum (pINN, USAN)	Antibiotic, produced by Streptomyces capreolus.
Carfenazinum (pINN USAN)	10-(3-(1-(2-Hydroxyethyl)piperazinyl-(4))-propyl)-2-propionylphenothiazine. USAN Carphenazine Maleate (dimaleate). Ⓡ Proketazine. (Psycho-sedative)
Cetoximum (BAN,pINN)	N-Benzylanilinoacet amidoxime. Ⓡ Febramine. (Antihistaminic)
Chloracyzinum (DCP,pINN)	-Chloro-10-(3-diethylaminopropionyl)phenothiazine. (Coronary dilator)

NFN name	*Other Names*
Chloralodolum (pINN)	2 Methyl-4-(2,2,2 trichloro-1-hydroxyethoxy)-pentanol-(2). BAN Chlorhexadol. ® Lora, Mechloral, Mecoral. (Hypnotic)
Chlormadinonum acetic acid ester chlormadinoni acetas (pINN USAN)	6-Chloro-17α-hydroxy 3 20-dioxopregnadiene-(4,6) = 6-chloro-17α hydroxy-6-dehydroprogesterone. pINN Chlormadinoni acetas, USAN Chlormadinone Acetate (acetic acid ester). (Progestogen)
Chlorprednisonum 21 acetic acid ester chlorprednisoni acetas (pINN)	6α-Chloro-17α,21-dihydroxy 3 11,20-trioxo-pregnadiene-(1 4) = 6α-chloroprednisone. pINN Chloroprednisoni acetas (21 acetic acid ester). (Glucocorticoid)
Chlorprothazinum (DCF pINN)	2-Chloro-10-(3-diethylaminopropyl)phenothiazine. ® Neuriplege. (Antiparkinson agent)
Chlortalidonum (BAN pINN USAN)	2-Chloro-5-(1-hydroxy-3-oxoisoindolinyl-(1))-benzenesulfonamide. BAN USAN Chlorthalidone. ® Hygroton (Diuretic)
Cinitr nadum (pINN)	3 4 5-Trimethoxycinnamamide. (Psycho-sedative)
Cismadinonum acetic acid ester cismadinoni acetas (pINN USAN)	6α Chloro-17α-hydroxy 3,20-dioxopregnadiene-(1 4) = 6α-chloro-17α hydro y 1-dehydroprogesterone. pINN Cismadinoni acetas, USAN Cismadinone Acetate (acetic acid ester). (Progestogen)
Clofedanolum (pINN)	1-(? Chlorophenyl)-3-dimethylamino-1 phenylpropanol-(1) = 2-chloro-α-(2-dimethylaminoethyl)benzhydrol. BAN Chlophedianol. USAN Chlophedianol Hydrochloride (chloride). ® Detigon, Refugal, Ulo (Antitussive)
Clofenetaminum (DCF pINN)	1-(1-(4-Chlorophenyl)-1 phenylethoxy)-2-diethylaminoethane. ® Keithon. (Antiparkinson agent)
Clometocillinum (DCF pINN)	6-(2-(3 4-Dichlorophenyl)-2 methoxyacetamido)-3,3-dimethyl 7-oxo-4-thia 1-azabicyclo-[3,2,0]heptanecarboxylic acid-(?) = (3 4-dichloro-α methoxybenzyl)penicillin. ® Rixapen. (Antibiotic)
Clomifenum (BAN pINN USAN)	2-Chloro-1-(4-(2-diethylaminoethoxy)phenyl)-1 2-diphenylethene. BAN Clomiphene. USAN Clomiphene Citrate (dihydrogen citrate). (Gonadotrop-inhibitor)

NFN-name	*Other Names*
Clopenthixolum (pINN)	2-Chloro-9-[3-(4-(2-hydroxyethyl)piperazinyl-(1))propylidene]thiaxanthene. ® Sordinol. (Psycho-sedative)
Cloxestradiolum diacetic acid ester cloxestradioli acetas (pINN)	3-Hydroxy-17β-(2,2,2-trichloro-1-hydroxyethoxy)-estratriene-(1,3,5(10)). pINN Cloxestradioli acetas (diacetic acid ester). ® Genovul. (Oestrogen)
Cloxotestosteronum acetic acid ester cloxotestosteroni acetas (pINN)	3-Oxo-17β-(2,2,2-trichloro-1-hydroxyethoxy)-androstene-(4). pINN Cloxotestosteroni acetas (acetic acid ester). ® Caprosem. (Androgen)
Cyclobutyrolum (DCF,pINN)	2-(1 Hydroxycyclohexyl)butyric acid. ® Hebucol. (Choleretic)
Cyclopenthiazidum (BAN,pINN,USAN)	6-Chloro-3-cyclopentylmethyl-7-sulfamoyl-3,4-dihydro-2H-1,2,4-benzothiadiazinedioxide-(1 1). ® Navidrex, Navidrix. (Diuretic)
Cyclopyrronium bromide cyclopyrronii bromidum (pINN USAN)	1 Ethyl-1-methyl-3-(cyclopentylphenylacet-oxy)pyrrolidiniumhydroxide. pINN Cyclopyrronii bromidum, USAN Cyclo-pyrronium Bromide (bromide). (Anticholinergic)
Cyclothiazidum (pINN,USAN)	6-Chloro-3-(norbornen-(5)-yl-(2))-7-sulfamoyl-3 4-dihydro-2H-1,2,4-benzothiadiazinedioxide-(1 1). ® Anhydron. (Diuretic)
Defosfamidum (pINN)	N N-Bis(2-chloroethyl)-N -(3-hydroxypropyl)-phosphorodiamidic acid (2-chloroethyl) ester ® Mitarson (Antineoplastic)
Deptropinum (pINN)	3-(10,11 Dihydro-5H-dibenzo[a,d]cyclo-heptenyl-(5)-oxy)tropane. ® Brontina. (Anticholinergic)
Dextrothyroxinum sodium salt dextrothyroxinnatrium (BAN,pINN USAN)	D-2 Amino-3-(4-(4-hydroxy-3,5-di-iodophenoxy)-3,5-di-iodophenyl)propionic acid = D-3,3 ,5,5 tetra-iodothyronine. pINN Dextrothyroxinum natricum, USAN Sodium Dextrothyroxine (sodium salt). ® Choloxin, Dethyron, Dethyrona. (Against hypercholesterolaemia)
Diazepamum (BAN,pINN,USAN)	7-Chloro-1-methyl-2-one-5-phenyl-1,3-dihydro-2H-1 4-benzodiazepine. ® Valium. (Psycho-sedative)

NFN name	*Other Names*
Diazoxidum (BAN pINN USAN)	7-Chloro-3-methyl 1,2,4-benzothiadiazine dioxide-(1 1) (Antihypertensive)
Difencloxazinum (DCF pINN)	1-(4-Chlorodiphenylmethoxy)-2-morpholinoethane DCF Diphenchloxazine. ® Olympax. (Sedative, antihistaminic)
Dimethadionum (pINN)	5,5-Dimethyl-2,4-dioxooxazolidine. (Anticonvulsive)
Dioxamatum (BAN pINN)	(2 Methyl 2-nonyl 1,3-dioxolanyl-(4))-methanol carbamidic acid ester (Antiparkinson agent)
Diprofenum (pINN)	Diphenylthioacetic acid (2-dipropylaminoethyl)-ester (Vasodilator)
Droxipropinum (BAN pINN)	1 [1-(2-(2 Hydroxyethoxy)ethyl)-4-phenyl piperidyl-(4)]propanone-(1) BAN Droxypropine. pINN Droxypropinum. (Antitussive)
Dydrogesteronum (BAN DCF pINN USAN)	3 20-Dioxo-9β,10α pregnadiene-(4 6) = 6--dehydro-9β 10α progesterone. ® Duphaston, Du aron, Gestatron Terolut. (Progestogen)
Epioestriolum (BAN pINN)	3 16β,17β-Trihydroxyestratriene-(1,3,5(10)). pINN Epiestriolum. ® Actriol. (Against Acne vulgaris)
Epipropidinum (pINN USAN)	1 1 Di(2,3-epoxypropyl)-4 4 bipiperidine. ® Eponate. (Antineoplastic)
Etaminilum (pINN)	3-Cyano-5-dimethylamino-3-phenylhexane = 4-dimethylamino-2-ethyl-2 phenylvaleronitrile. (Antitussive)
Etamivanum (BAN pINN USAN)	N N Diethylvanillamide. BAN USAN Ethamivan. ® Emivan, Sovanil, Vandid. (Analeptic)
Etebenecidum (BAN pINN)	4-Diethylsulfamoylbenzoic acid BAN Ethebenecid. ® Urelim (Uricosuric)
Ethomoxanum (BAN,pINN)	(±)-2 Butylaminomethyl-8-ethoxy 1 4-benzodioxane. (Psycho-sedative)
Etryptaminum (BAN pINN USAN)	3-(2 Aminobutyl)indole. USAN Etryptamine Acetate (acetate). ® Monase. (Monoamine oxydase inhibitor)
Etybenzatropinum (USAN)	3-Diphenylmethoxy-8-ethylnortropane. pINN Etybenzatropinum. USAN Ethybenztropine. ® Ponalid. (Anticholinergic)

NFN-name	*Other Names*
Febarbamatum (pINN)	1-(3-Butoxy-2-carbamoyloxypropyl)-5-ethyl-2,4,6-trioxo-5-phenylhexahydropyrimidine = 1-(3-butoxy-2-carbamoyloxypropyl)-5-ethylbarbituric acid. Ⓓ Getryl. (Anticonvulsive)
Fenadiazolum (DCF pINN)	2-(2 Hydroxyphenyl)-1,3 4-oxadiazole. DCF Phénadiazole. Ⓓ Hypnazol. (Hypnotic)
Fenbutrazatum (BAN,pINN)	2 Phenylbutyric acid (2-(3-methyl-2-phenyl-morpholino)ethyl)ester BAN Phenbutrazate. (Anorexigenic)
Fencamfaminum (BAN,pINN)	3-Phenyl-N-ethylnorbornanamine-(2). Ⓓ Euvitol. (Psycho-analeptic)
Fenoxazolinum (DCF,pINN)	2-(2-Isopropylphenoxymethyl)-2-imidazoline. (Vasoconstrictor local anaesthetic)
Fenoxypropazinum (BAN,pINN)	(1 Methyl-2-phenoxyethyl)hydrazine. BAN Phenoxypropazine. pINN Fenoxypropazinum. Ⓓ Drazine. (M onoamine oxydase inhibitor)
Fenyramidolum (DCF pINN USAN)	1 Phenyl-2-(pyridyl-(2)-amino)ethanol-(1). USAN Phenyramidol Hydrochloride (chloride). Ⓓ Analexin. (Analgesic, anticonvulsive)
Ferristrenatrium	Iron (III)-chelate of the monosodium salt of N,N,N N' tetrakis(carbo ymethyl)ethylenediamine = Iron(III)-chelate and sodium salt of ethylenediaminetetraacetic acid. BAN Sodium Ironedetate. pINN Natrii feredetas. Ⓓ Ferrofer Sytron. (Treatment of iron deficiency)
Fludroxycortidum (pINN)	6α-Fluoro-11β,16α,17α,21-tetrahydroxy-3,20-dioxo-pregnene-(4)-16,17-acetonide = 6α-fluoro-16α-hydroxy-hydrocortisone-16,17-acetonide. BAN USAN Flurandrenolone. pINN Fludroxy-cortidum. ● Cordran, Drenison, Drocort. (Glucocorticoid)
Flunisolidum 21 acetic acid ester flunisolidi acetas (pINN,USAN)	6α-Fluoro-11β,16α,17α,21-tetrahydroxy-3,20-dioxopregnadiene-(1,4)-16,17-acetonide. pINN Flunisolidi acetas, USAN Flunisolide Acetate (21 acetic acid ester). (Glucocorticoid)
Fluoresonum (DCF,pINN)	Ethyl-(4-fluorophenyl)sulfone. Ⓓ Bripadon. (Antiepileptic)

NFN-name	*Other Names*
Fluroxenum (pINN USAN)	2,2,2 Trifluoroethyl vinyl ether ® Fluoromar (Anaesthetic)
Ftivazidum (pINN)	N Isonicotinoyl N vanillylidenehydrazine. (Chemotherapeutic)
Ganglefenum (pINN)	4-Isobutoxybenzoic acid (3-diethylamino-1,2 -dimethylpropyl)ester ® Ganglerone. (Anticholinergic)
Glycopyrronum bromide glycopyrroni *bromidum* (BAN pINN)	1 1 Dimethyl 3-(cyclopentylphenylglycoloyl-oxy)pyrrolidiniumhydroxide. BAN Glycopyrronium Bromide. pINN *Glyco-pyrronii* bromidum (bromide). ® Robinul, Tarodyl Tarodyn. (Anticholinergic)
Glycyclamid m (pINN)	1 Cyclohexyl 3-(tolyl-(4)-sulfonyl)carbamide. ® Cyclamide. (Antidiabetic)
Glyzobuzolum (DCF pINN)	N-(5-Isobutyl 1,3 4-thiadiazolyl-(2))-4--methoxybenzenesulfonamide. BAN Isobuzole. ® Stabinol. (Antidiabetic)
Haletazolum (BAN pINN)	5-Chloro-2-(4-(-diethylaminoethoxy)phenyl)-benzothiazole. BAN Halethazole. ® Episol (Fungicide)
Heptolamidum (pINN USAN)	I Cycloheptyl-3-(totyl-(4)-sulfonyl)carbamide. (Antidiabetic)
Hexafluronum bromide hexafluroni bromidum (pINN)	Hexamethylenbis(fluorenyl-(9)-dimethyl ammoniumhydroxide). pINN Hexafluronii bromidum, USAN Hexa fluorenium Bromide (bromide). ® Mylaxen. (Curarising)
Hexaprodol m (pINN)	(1 Aminohexyl)diphenylmethanol = α-(1 aminohexyl)-benzhydrol. (Psycho-analeptic)
Hydromadinon m acetic acid ester hydromadinoni acetas (pINN USAN)	6α-Chloro-17α-hydroxy-3 20-dioxopregnene--(4) = 6α-chloro-17α-hydro yprogesterone. pINN Hydromadinoni acetas, USAN Hydromadinone Acetate (acetic acid ester) (Progestogen)
Indopinum (pINN USAN)	3-(2-(1 Phenethylpiperidyl-(4))ethyl)indole. USAN Indopine Hydrochloride (chloride) (Analgesic, sedative)

NFN-name	*Other Names*
Inositoli nicotinas (pINN)	Inositol hexanicotinic acid ester USAN Inositol Niacinate. Ⓓ Hexanicit, Linodil. (Vasodilator)
Interferonum (BAN,pINN)	A protein formed by the interaction of animal cells with viruses capable of conferring on animal cells resistance to virus infection.
Isopropicillinum (pINN USAN)	3,3-Dimethyl-6-(2-methyl-2 phenoxypropionamido)-7-oxo-4-thia-1-azabicyclo[3,2,0]heptanecarboxylic acid-(2) = α-phenoxyisopropylpenicillin. USAN Potassium Isopropicillin (potassium salt). (Antibiotic)
Iofendylatum (BAN,pINN,NND)	10-(4-Iodophenyl)undecanoic acid ethyl ester BAN, NND Iophendylate. pINN Iofendylatum. Ⓓ Ethiodan, Myodil, Pantopaque. (X-ray contrast medium)
Laurolinium acetate laurolinii acetas	4-Amino-1-dodecylquinaldinium hydroxide. BAN Laurolinium Acetate, pINN Laurolinii acetas (acetate). Ⓓ Laurodin. (Antibacterial)
Levamphetaminum (pINN USAN)	(—)-2-Amino-1-phenylpropane. pINN Levamfetaminum. USAN Levamfetamine Succinate (succinate). Ⓓ Cydril. (Sympatomimetic)
Levopropicillinum (pINN)	(—)-3,3-Dimethyl-7-oxo-6-(2-phenoxybutyramido)-4-thia-1-azabicyclo[3,2,0]heptanecarboxylic acid-(2) = (—)-α-phenoxypropylpenicillin. USAN Potassium Levopropylcillin (potassium salt). (Antibiotic)
Mebutamatum (BAN,pINN,USAN)	2 Sec-butyl-2-methylpropanediol-(1,3) dicarbamidic acid ester Ⓓ Capla, Prandiex. (Psycho-sedative)
Mecloqualonum (DCF pINN)	3-(2-Chlorophenyl)-2-methyl-3H-quinazolinone-(4). (Hypnotic, antitussive)
Mefeclorazinum (DCF,pINN)	1-(2-Chlorophenyl)-4-(3 4-dimethoxyphenethyl)-piperazine. DCF Méphéchlorazine. (Psycho-sedative)
Melarsonylum (BAN,DCF pINN)	2-[4-((4 6-Diamino-s-triazinyl-(2))amino)phenyl]-1,3,2-dithiarsolanedicarboxylic acid-(4,5). BAN Melarsonyl Potassium, DCF Mélarsonyl potassique, pINN Melarsonylum kalicum (potassium salt). Ⓓ Trimelarsan. (Chemotherapeutic)

NFN name	*Other Names*
Meractinomycinum (pINN)	Actinomycin D (Antineoplastic)
Mestranolum (BAN DCF pINN USAN)	17α Ethynyl-17β-hydroxy 3-methoxyoestratriene-(1,3,5(10)) (Oestrogen)
Metacyclinum (BAN pINN USAN)	4-Dimethylamino-3 5 10 12,12a pentahydroxy-6-methylene-1 11-dioxo-1 4 4a,5 5a,6,11 12a-octahydronaphthacenecarboxamide-(2) BAN USAN Methacycline. ® Rondomycin. (Antibiotic)
Metaglycodolum (pINN)	2-(3-Chlorophenyl)-3-methylbutanediol-(2,3) (Psycho-sedative)
Metamfazonum (BAN DCF pINN)	4-Amino-6-methyl-2 phenyl 2H pyridazinone-(3). BAN (DCF) Methamphazone. (Analgesic)
Metandienonum (BAN pINN)	17β-Hydroxy 17α methyl-3-oxoandrostadiene-(1 4). BAN Methandienone. USAN: Methandrostenolone. ® Dianabol. Geabol Vanabol. (Anabolic, androgen)
Metenolonum (BAN pINN)	17β-Hydroxy 1 methyl-3-oxo-5α-androstene-(1). BAN Methenolone. ® Primobolan, Primobolan-depot. (Anabolic, androgen)
Metetoinum (BAN pINN USAN)	5 Ethyl-1 methyl 2,4-dioxo-5-phenylimidazoline = 5-ethyl-1 methyl 5-phenylhydantoine. BAN USAN Methetoin (Antiepileptic)
Methyldopum (BAN DCF pINN USAN)	L 3-(3 4-Dihydroxyphenyl)-2 methylalanine. Metyldopa. BAN (DCF) USAN Methyldopa. ® Aldomet (Antihypertensive)
Meticillinum (BAN DCF pINN USAN)	6-(2,6-Dimethoxybenzamido)-3 3-dimethyl 7-oxo-4-thia 1 azabicyclo[3,2,0]heptanecarboxylic acid-(2) = (2,6-dimethoxyphenyl)penicillin. BAN Methicillin DCF Méthicilline sodique. USAN Sodium Methicillin (sodium salt). ® Belfacillin, Celbenin Dimocillin Lucopenin, Stafylopenin, Staphcillin Synticillin (Antibiotic)
Metofolinum (pINN USAN)	1-(4-Chlorophenethyl)-6,7-dimetho y 2 methyl 1,2,3 4-tetrahydroisoquinoline USAN Methopholine Hydrochloride (chloride). (Analgetic)
Morazonum (BAN pINN)	2,3-Dimethyl-4-((3 methyl 2 phenylmorpholino)-methyl)-1 phenylpyrazolone-(5) = 4-((3-methyl-2 phenylmorpholino)methyl)antipyrine. (Analgetic)

NFN-name	*Other Names*
Moroxydinum (BAN,pINN USAN)	4-Morpholinecarboximidoylguanidine. (Antiviral)
Nicocodinum (BAN,pINN)	(−)-3-Methoxy-17-methyl-6-nicotinoyloxy-4,5-epoxymorphinene-(7) = 6-nicotinoylcodeine. (Antitussive)
Noracymethadolum (BAN,pINN)	Acetic acid ((±)-6-methylamino-4,4-diphenyl-heptanyl-(3))ester (*Analgetic, euforiadog*)
Nortriptylinum (BAN,pINN,USAN)	5-(3-Methylaminopropylidene)-10,11-dihydro-5H-dibenzo[a,d]cycloheptene. USAN Nortriptyline Hydrochloride (chloride). ® Aventyl Hydrochloride. (Psycho-analeptic)
Noxytiolinum (BAN pINN)	1 Hydroxymethyl-3-methyl-2-thiocarbamide. BAN Noxythiolin. pINN Noxytiolinum. (Antibacterial)
Opiniazidum (pINN)	2,3-Dimethoxy-6-isonicotinoylhydrazono-methylbenzoic acid = isonicotinoyl opianic acid hydrazone. ® Saluside. (Chemotherapeutic)
Oxandrolonum (pINN USAN)	3-Hydroxy-6-hydroxymethyl-3,3a,6-trimethyl-*dodecahydro*-1H-benz[e]indene-acetic acid-(7)-lactone = 17β-hydroxy-17α-methyl-3-oxo-2-oxa-5α-androstane. ® Anavar (Anabolic, androgen)
Oximesteronum (BAN,DCF,pINN)	4,17β-Dihydroxy-17 -methyl-3-oxoandrostene-(4). BAN, (DCF) Oxymesterone. pINN Oxymesteronum. ® Oranabol. (Anabolic, androgen)
Oxypertinum (pINN,USAN)	5,6-Dimethoxy-2-methyl-3-(2-(4-phenylpiperazinyl-(1))ethyl)indole. pINN Oxypertinum. USAN Oxypertine Hydro-chloride (chloride). (Psycho-sedative)
Paramethasonum (BAN,DCF pINN,USAN)	6α-Fluoro-11β,17α,21-trihydroxy-16α-methyl-3,20-dioxopregnadiene-(1 4) = 6α-fluoro-16α-methyl-prednisolone. USAN Paramethasone Acetate (21 acetic acid ester). ® Dilar Haldrate, Haldrone, Metilar (Glucocorticoid)
Pemolinum (BAN,pINN)	2-Imino-5-phenyloxazolidinone-(4). ® Hyton, Kethamed, Pioxol, Ronyl, Tradon. (Psycho-analeptic)
Penicillaminum (BAN,pINN)	D-3-Mercaptovaline. BAN D-Penicillamine. (Antineoplastic)
Pentamoxanum (pINN)	2 Isopentylaminomethyl-1 4-benzodioxane. (Psycho-sedative)

NFN name	*Other Names*
Pipacyclinum (pINN)	4-Dimethylamino-3 6 10 12,12a pentahydroxy-N-[(4-(2 hydroxyethyl)piperazinyl-(1))methyl]-6-methyl-1 11-dioxo-1 4 4a,5,5a,6,11 12a-octahydronaphthacene-carboxamide-(2) = N [(4-(2 hydroxyethyl)piperazinyl-(1))methyl]tetracycline. ® Ambraveln Valtomicina. (Antibiotic)
Piparetatum (BAN DCF pINN)	10H Pyrido[3,. b][1 4]benzothiazinecarboxylic acid-(10)(2-(2 piperidinoethoxy)ethyl)ester BAN Pipazethate. ® Lenopect, Selvigon Theratuss. (Antitussive)
Polynoxylinum (BAN pINN)	Poly(methylenebis(hydroxymethyl)carbamide). ® Anaflex. (Antibacterial)
Polythiazidum (BAN pINN USAN)	6-Chloro-2 methyl-7-sulfamoyl 3-((2,2,2-trifluoroethyl)thiomethyl)-3 4-dihydro-.H 1,2,4-benzothiadiazinedioxide-(1 1). ® Renese. (Diuretic)
Prenylaminum (BAN pINN USAN)	-(3,3-Diphenylpropylamino)-1 phenylpropane. ® Segontin, Synadrin (Coronary vasodilator)
Pridinolum (pINN)	1 1 Diphenyl-3-piperidinopropanol-(1). ® Ridinol. (Antiparkinson agent)
Pristinamycinum (DCF pINN)	Antibiotic, produced by Streptomyces pristina spiralis.
Prodilidinum (pINN USAN)	Propionic acid (1,2-dimethyl-3 phenylpyrrolidinyl-(3))ester USAN Prodilidine Hydrochloride (chloride). ® Cogesic. (Analgesic)
Propatylnitratum (BAN DCF pINN)	Ethyl -(hydro ymethyl)propanediol-(1,3)-trinitric acid ester USAN Propatyl Nitrate. ® Etrynit, Gina. (Vasodilator)
Propinetidinum (pINN)	Propionic acid (1 phenethyl-4-(propyn-( )-yl)-piperidyl-(4))ester (Antitussive)
Propyromazinum (DCF pINN)	1 Methyl 1-(1 phenothiazin yl-(10)-carbonyl-ethyl)pyrrolidinium hydroxide (Anticholinergic, ganglion blocking)
Psilocybinum (DCF pINN)	Phosphoric acid mono-(3-(2-dimethylami oethyl)-indolyl-(4))ester ® I docybin. (Psycho-somimetic)

NFN-name	*Other Names*
Pyritinolum (pINN)	Bis((5-hydroxy-4-hydroxymethyl-6-methyl-pyridyl-(3))methyl)disulfide. ® Bonifen. (Psycho-pharmakon)
Rofluranum (pINN,USAN)	(2-Bromo-1,1,2-trifluoroethyl)methyl ether (Anaesthetic)
Rufocromomycinum (DCF,pINN)	Antibiotic, produced by Streptomyces rufochromogenus. (Antineoplastic)
Secumalum	5-Sec-butyl-5-ethyl-2,4,6-trioxohexahydropyrimidine = 5-sec-butyl-5-ethylbarbituric acid. DCF Secbutabarbital sodique (sodium salt). pINN Secbutabarbitalum. USAN Butabarbital Sodium (sodium salt). ® Butisol, Paxital. (Hypnotic)
Solypertinum (pINN USAN)	7-[2-(4-(2-Methoxyphenyl)piperazinyl-(1))ethyl]-5H-1,3-dioxolo[4,5-f]indole. USAN Solypertine Tartrate (tartrate). (Sympatolytic)
Spir[illegible] (pINN)	2,4-Diamino-5-(4-chlorophenyl)-9-methyl-1,3,5-triazaspiro[5,5]undecadiene-(1,3). (Anthelmintic)
Sulfacarbamidum (pINN)	Sulfanilylcarbamide. BAN Sulphaurea. ® Euvernil. (Chemotherapeutic)
Sulfalenum (pINN)	3-Methoxy-2-sulfanilamidopyrazine. ® Kelfizina. (Chemotherapeutic)
Sulfametomidinum (pINN)	6-Methoxy-2-methyl-4-sulfanilamidopyrimidine. ® Methofadin. (Chemotherapeutic)
Sulfamoxolum (BAN,pINN USAN)	4,5-Dimethyl-2-sulfanilamidooxazole. BAN Sulphamoxole. ® Nuprin, Sulfune, Sulfuno, Tardamid. (Chemotherapeutic)
Sulf[illegible] (pINN)	4,6-Diethyl-2-sulfanilamido-s-triazine. (Chemotherapeutic)
Symetinum (pINN USAN)	4,4'-(Ethylenedioxy)bis(N-hexyl-N-methyl-benzylamine). USAN Symetine Hydrochloride (chloride). (Amoebicide)
Teclothiazidum (BAN DCF,pINN)	6-Chloro-7-sulfamoyl-3-trichloromethyl-3,4-dihydro-2H 1,2,4-benzothiadiazine-dioxide-(1,1). ® Depleil, Depiet. (Diuretic)
Tefluranum (pINN)	2-Bromo-1,1,1,2-tetrafluoroethane. (Anaesthetic)

NFN name	*Other Names*
Pipacyclinum (pINN)	4-Dimethylamino-3 6,10 12,12a-pentahydroxy-N-[(4-(2 hydroxyethyl)piperazinyl-(1))methyl]-6-methyl-1 11-dioxo-1 4 4a,5,5a,6,11 12a-octahydronaphthacene-carboxamide-(2) = N [(4-(2 hydroxyethyl)piperazinyl-(1))methyl]tetracycline. ® Ambravcin, Valtomicina. (Antibiotic)
Pipazetatum (BAN DCF pINN)	10H Pyrido[3 2 b][1 4]benzothiazinecarboxylic acid-(10)(2-(2 piperidinoethoxy)ethyl)ester BAN Pipazethate. ® Lenopect, Selvigon, Theratuss. (Antitussive)
Polynoxylinum (BAN pINN)	Poly(methylenebis(hydroxymethyl)carbamide). ® Anaflex. (Antibacterial)
Polythiazidum (BAN pINN USAN)	6-Chloro-2-methyl-7 sulfamoyl 3-((2,2,2-trifluoroethyl)thiomethyl)-3 4-dihydro-2H 1 ,4-benzothiadiazinedioxide-(1 1). ® Renese. (Diuretic)
Prenylaminum (BAN pINN USAN)	2-(3 3-Diphenylpropylamino)-1-phenylpropane. ® Segontin, Synadrin (Coronary vasodilator)
Pridinolum (pINN)	1 1 Diphenyl 3-piperidinopropanol-(1). ® Ridinol. (Antiparkinson agent)
Pristinamyci m (DCF pINN)	Antibiotic, produced by Streptomyces pristina spiralis.
Prodilidinum (pINN USAN)	Propionic acid (1,2-dimethyl 3-phenylpyrrolidinyl-(3))ester USAN Prodilidine Hydrochloride (chloride). ® Cogesic. (Analgesic)
Propatylnitratum (BAN DCF pINN)	2 Ethyl 2-(hydroxymethyl)propanediol-(1 3)-trinitric acid ester USAN Propatyl Nitrate. ® Etrynit, Gina. (Vasodilator)
Propinetidin m (pINN)	Propionic acid (1 phenethyl-4-(p pyn-(?)-yl)-piperidyl-(4))ester (Antitussi e)
Propyromazinum (DCF,pINN)	1 Methyl-1-(1 phenothiazinyl-(10)-carbonyl ethyl)pyrrolidiniumhydroxide. (Anticholinergic, gangli blocking)
Psilocybinum (DCF pINN)	Phosphoric acid mono-(3-(2-dimethylaminoethyl)-indolyl-(4))ester ® Indocybin (Psycho-somimetic)

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The Effect of a β-Adrenergic Blocking Agent (Nethalide[1]) in Vitro on the Metabolism of Adipose Tissue

By

Per Björntorp

(Received March 19 1964)

In the search for a β-adrenergic blocking agent, 2 isopropylamine 1 (2 naphthyl) ethanol hydrochloride (nethalide ®) was synthesized and found to produce an effective blockage of myocardial adrenergic receptors (BLACK & STEPHENSON, 1962) without adverse physiological effects. The drug was in this investigation found to be free from sympathicomimetic activity

SCHRÖDER & BJÖRNTORP (1964) found a change in free fatty acid pattern after work load in human subject taking nethalide. One possible explanation to this was an effect of Nethalide on the metabolism of adipose tissue. Adrenaline and its analogues have pronounced effects on such metabolism. They increase fatty acid output from adipose tissue (GORDON & CHERKES 1958) in spite of an increase in glucose uptake and $^{14}CO_2$ production from glucose 6-^{14}C which gives an increase of fatty acid esterification to glycerol-phosphate (CAHILL *et al* 1960). The effect on fatty acid output is thus not brought about by a decrease in triglyceride synthesis, but by a true increase in lipolysis, as indicated by an output also of glycerol from adipose tissue (LEBOEUF *et al* 1959). The trigger mechanism for this appears to be a lipase, whose activity has been shown to be stimulated by adrenaline (RIZACK 1961 BJÖRNTORP & FURMAN 1962 BJÖRNTORP 1964)

Because of the sensitivity of adipose tissue to adrenaline, it was thought of interest to study the effect of nethalide on the stages of carbohydrate and lipid metabolism in adipose tissue *in vitro* already mentioned.

[1] Imperial Chemical Industries Limited (I.C.I. 38, 174). Also called "Pronethalol" and Alderlin

NFN name	*Other Names*
Thenl closylas (pINN USAN)	N N Dimethyl N-(2 phenoxyethyl)-N 2-tenylammonium salt of 4-chlorobenzenesulfonic acid. pINN Thenil closylas. USAN Thenium Closylate. ® Bancaris. (Anthelmintic)
Thiohexamidum (pINN)	1 Cyclohexyl-3-((4-methylthiophenyl)sulfonyl)carbamide. (Antidiabetic)
Tifencillinum (pINN USAN)	3,3-Dimethyl 7-oxo-6-(2 phenylthioacetamido)-4-thia 1-azabicyclo[3,2,0]heptanecarboxylic acid-(2) = (phenylthiomethyl)penicillin. USAN Potassium Thiphencillin (potassium salt). (Antibiotic)
Tolazamid m (pINN)	1-(Hexahydro-1H-azepinyl-(1))-3-(tolyl-(4)-sulfonyl)carbamide) (Antidiabetic)
Tolpentamidum (BAN pINN)	1-Cyclopentyl 3-(tolyl-(4)-sulfonyl)carbamide. (Antidiabetic)
Tozalino um (pINN USAN)	2 Dimethylamino-4-oxo-5-phenyl 2-oxazoline. USAN Thozalinone. (Psycho-analeptic)
Triamterenum (BAN pINN USAN)	2,4 7 Triamino-6-phenylpteridine. (Diuretic)
Trimedoximum bromide trimedoximi bromidum (pINN)	1 1 Trimethylenebis(4-formylpyridinium-hydroxide)dioxime. (Anticholinesterase antagonist)
Vinblastinum (BAN pINN USAN)	Vincaleukoblastin, alkaloid isolated from Vinca rosea. USAN Vinblastine Sulfate (sulfate). ® Velban Velbe. (Antineoplastic)
Vinymalum	5-(1 Methylbutyl)-2,4 6-trioxo-5-vinylhexahydropyrimidine = 5-(1 methylbutyl)-5-vinylbarbituric acid. BAN Vinylbitone. pINN Vinylbitalum (Hypnotic)
Xanthiolum (pINN)	2 Chloro-9-[3-(4-(3-hydroxypropyl)piperazinyl)propyl]thiaxanthene. ® Daxid. (Psycho-sedative)
Xantocillinum (BAN pINN)	Antibiotic, produced by Penicillium notatum. BAN Xanthocillin.
Xenysalatum (BAN pINN)	3-Phenylsalicylic acid (2-diethylaminoethyl) ester USAN Biphenamine Hydrochloride (chloride). (Antibacterial)
Zylofuraminum (pINN USAN)	(+)-Threo-2-(1-ethylamino-2 phenylethyl)-tetrahydrofurane = (+)-threo-α benzyl-N-ethyltetrahydrofurfurylamine. USAN Zylofuramine Hydrochloride (chloride). (Psycho-analeptic)

Table I

Effects of Adrenaline and Nethalide on Fatty Acid and Glycerol Release, Lipase Activity and Glucose Uptake in Rat Epididymal Fat Pads Incubated in Vitro. Mean ± Standard Deviations. In parentheses Number of determinations.

	0	Nethalide 5×10^{-6}M	Adrenaline 5×10^{-6}M	Adrenaline + Nethalide 5×10^{-6}M 5×10^{-6}M
Fatty acid released (μEq/g)	2.70 ±0.46 (6)	4.32 ±0.40 (5)	7.62 ±0.66 (5)	8.98 ±0.66 (6)
Glycerol released (μEq/g)	1.30 ±0.20 (6)	2.60 ±0.40 (5)	7.00 ±0.53 (6)	6.45 ±0.52 (6)
Lipase activity (μEq FA/g/h)	6.45 ±0.91 (4)	8.52 ±0.88 (4)	10.19 ±0.62 (4)	10.03 ±1.33 (4)
Glucose uptake (mg/g)	1.86 ±0.46 (6)	1.60 ±0.40 (5)	4.46 ±0.61 (6)	2.32 ±0.44 (6)

Nethalide stimulated fatty acid ($p < 0.01$) release and lipase activity ($p < 0.05$) in the absence of adrenaline from the flask. Adrenaline gave a more pronounced stimulation ($p < 0.001$), and the addition of nethalide to adrenaline gave a further increase in fatty acid release ($p < 0.02$), but not in lipase activity.

Glycerol release followed the pattern of lipase activity *viz.* an increase after nethalide only ($p < 0.01$), a further increase with adrenaline only ($p < 0.001$) and *no increase in release by adrenaline after adding* nethalide. Glucose uptake showed no significant changes after nethalide. It increased after adrenaline ($p < 0.001$) and again decreased in the adrenaline plus nethalide flask, compared with adrenaline alone ($p < 0.001$). These effects are also shown in fig. 1 where the approximate linearity of the time-activity curves for 3 hours is seen.

The effects of increasing concentrations of nethalide on the metabolism of adipose tissue stimulated by a 5×10^{-6}M concentration of adrenaline

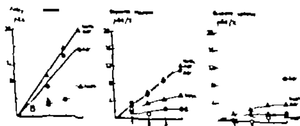


Fig. 1. Effect of Adrenaline and Nethalide on Rat Epididymal Fat Pads in Vitro. Concentrations of Drugs 5×10^{-6}M.

Experimental.

200–250 g male rats of the Wistar strain were beheaded, distal parts of the epididymal fat pads being then removed and placed in Ringer s solution at room temperature. They were then immediately cut into pieces, which were randomized, weighed and incubated.

The incubation medium for fatty acid and glycerol release and glucose measurement consisted of 5 ml Krebs-Ringer phosphate buffer with 4 / serum albumin (Armour Fraction V). The final pH was 6 8 Glucose, when present, was at a concentration of 5 6 mM Incubations were performed in a Dubnoff type incubator at 37 4°C with air as gas phase. Free fatty acids, glycerol and glucose were determined at 0 and 2 hours.

Lipase activity was assayed in a system consisting of equal parts of enzyme, obtained by homogenizing the fat pad in a Potter Elvehjelm apparatus in Krebs-Ringer phosphate buffer and subsequent collection of the turbid water phase between the top fat cake and the sediment after centrifugation (BJÖRNTORP & FURMAN 1962), and a mixture of such composition as to give the final concentrations serum albumin, 4 KH_2PO_4 Na_2HPO_4(1 1), 0.005 M NaCl 0 6 M Ediol[1]) 0 8 / (concentration of triglycerides) Incubations were at 37°C and fatty acids were determined at 0 and 2 hours.

Incubation for measuring $^{14}CO_2$ and labelled lipid from glucose 6-^{14}C was performed in 2 ml of Krebs Ringer bicarbonate buffer pH 7 4 in the presence of 11.2 mM glucose. The vessels used were 50 ml cylindrical flat bottom tubes (Hagedorn tubes) sealed with a rubber stopper holding two glass tubes closed by rubber membranes. After 2 hours of incubation at 37°C, 0.3 ml Hyamine[2]) was injected through one of the glass tubes into a 1 ml beaker held in a stainless steel wire loop Through the other glass tube 0.2 ml of 10 N H_2SO_4 was then injected into the medium. After 4 hours, recovery of $^{14}CO_2$ in the beaker with Hyamine was complete, as tested in separate experiments with $NaH^{14}CO_3$ The beaker was then placed in 10 ml scintillation solution (0.4 / PPO[3]) and 0.01 / dimethyl POPOP[4]) in toluene) in a glass vial and radioactivity counted in a Packard Tri-Carb liquid scintillation counter

Incorporation of label into lipids was measured in the system mentioned. After incubation the tissue was extracted as described by FOLCH *et al* (1954) The chloroform phase was washed twice with water the last washings being practically free from radioactivity Finally a measured portion of the chloroform solution was evaporated in the counting vial, and 10 ml scintillation solution were added Counting was then performed as described above.

Glucose was determined enzymatically (LEVIN & LINDE 1962) glycerol by periodate oxidation as described by CARLSON & WADSTRÖM (1959) and fatty acids by the method of DOLE (1956) with Nile Blue as indicator

Statistical significance was assessed by the (student s) t test

Results

The effects on fatty acid release, lipase activity glycerol release and glucose uptake at $5 \times 10^{-6}M$ concentration of both adrenaline and nethalide are shown in table I

1) Riker Laboratories, Inc. (SchenLabs), Northridge, California, USA
2) p-(diisobutyl-cresoxyethoxyethyl)dimethylbenzylammonium Hydroxide (Rohm & Haas)
3) 2,5-diphenyloxazol (Packard)
4) 1 4-bis-[2-(4-methyl-5-phenyloxazolyl)-benzene] (Packard)

Table 1

Effects of Adrenaline and Nethalide on Fatty Acid and Glycerol Release, Lipase Activity and Glucose Uptake in Rat Epididymal Fat Pads Incubated in Vitro. Mean ± Standard Deviations. In parentheses Number of determinations.

	0	Nethalide 5×10^{-4}M	Adrenaline 5×10^{-6}M	Adrenaline + Nethalide 5×10^{-4}M 5×10^{-6}M
Fatty acid released (uEq/g)	2.70 ±0.46 (6)	4.32 ±0.40 (5)	7.62 ±0.66 (5)	8.98 ±0.66 (6)
Glycerol released (uEq/g)	1.30 ±0.20 (6)	2.60 ±0.40 (5)	7.00 ±0.53 (6)	6.45 ±0.52 (6)
Lipase activity (uEq FA/g/h)	6.45 ±0.91 (4)	8.52 ±0.88 (4)	10.19 ±0.62 (4)	10.03 ±1.33 (4)
Glucose uptake (mg/g)	1.86 ±0.46 (6)	1.60 ±0.40 (5)	4.46 ±0.61 (6)	2.32 ±0.44 (6)

Nethalide stimulated fatty acid ($p < 0.01$) release and lipase activity ($p < 0.05$) in the absence of adrenaline from the flask. Adrenaline gave a more pronounced stimulation ($p < 0.001$), and the addition of nethalide to adrenaline gave a further increase in fatty acid release ($p < 0.02$) but not in lipase activity

Glycerol release followed the pattern of lipase activity *viz.* an increase after nethalide only ($p < 0.01$), a further increase with adrenaline only ($p < 0.001$), and no increase in release by adrenaline after adding nethalide. Glucose uptake showed no significant changes after nethalide. It increased after adrenaline ($p < 0.001$) and again decreased in the adrenaline plus nethalide flask, compared with adrenaline alone ($p < 0.001$). These effects are also shown in fig. 1 where the approximate linearity of the time activity curves for 3 hours is seen.

The effects of increasing concentrations of nethalide on the metabolism of adipose tissue stimulated by a 5×10^{-6}M concentration of adrenaline

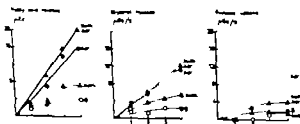


Fig. 1 Effect of Adrenaline and Nethalide on Rat Epididymal Fat Pads *in Vitro* Concentrations of Drugs 5 10^{-4}M

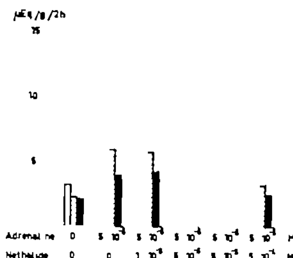


Fig. 2. Effects of Adrenaline and Nethalide on Metabolism of Rat Epididymal Fat Pads in Vitro Open columns Fatty Acid Release. Hatched columns Glycerol Release. Solid columns Glucose Uptake.

are shown in fig. 2. At a 5×10^{-6}M concentration of nethalide a decrease in glucose uptake was seen. Here fatty acid release increased. Glycerol release did not seem to be affected, however before nethalide concentration reached 5×10^{-5}M With this concentration fatty acid release also fell abruptly

The results of conversion of glucose 6-^{14}C to $^{14}CO_2$ and lipids are seen in table 2.

Adrenaline increased both incorporations ($p < 0.01$) Nethalide increased conversion to $^{14}CO_2$ ($p < 0.02$) Incorporation into lipids was significantly increased only at 5×10^{-6}M concentration ($p < 0.01$) Both concentrations markedly blocked the adrenaline effect ($p < 0.01$)

Table 2

Effects of Adrenaline and Nethalide on Conversion of Glucose 6-^{14}C to $^{14}CO_2$ and Lipid ^{14}C by Rat Epididymal Fat Pads. Means ± Standard Deviations of 6 Determinations.

	0	Adrenaline 10^{-6}M	Nethalide		Adrenaline (10^{-6}M) + Nethalide	
			5×10^{-6}M	5×10^{-5}M	5×10^{-6}M	5×10^{-5}M
CO_2	0.10 ±0.04	0.43 ±0.12	0.26 ±0.19	0.27 ±0.09	0.15 ±0.10	0.09 ±0.04
Lipid	0.44 ±0.11	0.79 ±0.14	0.73 ±0.12	0.33 ±0.09	0.39 ±0.10	0.09 ±0.03

Results expressed as μmol glucose-6-^{14}C converted to $^{14}CO_2$ or lipid-^{14}C per gram (wet weight) tissue per hour

Discussion

Thus it was found that nethalide diminished both adrenaline induced glucose uptake and lipolysis in adipose tissue *in vitro*

A decrease in glucose uptake diminishes fatty acid reesterification affecting fatty acid output, since glucose is the triglyceride-glycerol precursor in adipose tissue (VAUGHAN 1961). Glycerol once liberated from triglycerides does not seem to be able to take part in the re-esterification of fatty acids, because its activation is not considered possible in adipose tissue in the absence of glycerol kinase (WIELAND & SUYTER 1957). Its diffusion out of the tissue thus reflects lipolysis.

The parallel changes of glycerol release from the tissue and lipase activity seem to support the hypothesis that the lipase in question provides the trigger mechanism for lipolysis in adipose tissue (RIZACK 1961 BJÖRNTORP & FURMAN 1962)

With these concepts in mind, the nethalide effect on adrenaline stimulated metabolism could be interpreted as follows. Glucose uptake is inhibited at somewhat lower concentrations than is lipolysis. This first brings about an increase in fatty acid output and then, at higher concentrations, lipolysis is also inhibited, and fatty acid output consequently decreases abruptly Nethalide alone stimulates glycerol release.

This investigation thus tends to show that the increase in glucose uptake effected by adrenaline on adipose tissue can be inhibited at concentrations of nethalide lower than those interfering with the adrenaline lipolytic effect. This seems to indicate that the adrenaline effect on glucose uptake is not necessarily only a consequence of its effect on lipolysis and the accumulation of fatty acids (CAHILL *et al* 1960).

FRÖBERG & ORÖ (1963) recently studied the effect of nethalide on the adrenaline-induced changes of free fatty acid concentration in the arterial plasma of the dog. They found an increase in free fatty acid concentration after infusion of nethalide and a decrease in adrenaline response thereafter These results seem to correspond to the in Vitro findings in our work of a lipolytic effect of nethalide and a blockage of adrenaline-induced lipolysis.

The changes after nethalide on free fatty acid concentration of human plasma (SCHRÖDER & BJÖRNTORP 1964) could possibly be explained from the results obtained. The free fatty acid pattern during and after work load is dependent on lipolysis of adipose tissue (presumably induced by cathecholamines), utilization of free fatty acids and distribution of blood flow The free fatty acid peak immediately after work is probably caused by a continuing increase in lipolysis despite the cease in utilization (HAVEL *et al.* 1963). This peak has been observed to diminish after treatment with nethalide at high doses (SCHRÖDER & BJÖRNTORP 1964), which could be caused by blockage of lipolysis in adipose tissue, as shown possible with nethalide *in vitro*

Summary

Nethalide was found to diminish the effects of adrenaline on adipose tissue metabolism The increase in glucose uptake was found to be abolished at approximately equivalent concentrations of adrenaline and nethalide At higher concentrations lipolysis was decreased as indicated by a decrease in glycerol release Fatty acid release at rising concentrations of nethalide first increased after the effect of nethalide on glucose uptake and then fell abruptly after the effect also on lipolysis at higher concentrations This could be explained by interference between glycolysis and lipid metabolism in adipose tissue.

Nethalide alone seemed to excert a small stimulating action on lipolysis.

Acknowledgements.

The technical assistance of Miss Majvor Karlsson is gratefully acknowledged.

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Acetylcholine, Histamine, 5-Hydroxytryptamine and Catecholamine Contents of Mammalian Penile Erectile and Urethral Tissue

By

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The erectile tissue of the mammalian penis is formed of elastic fibres, unstriated muscle and arteries and veins of different size, all surrounded by a collagenous tunica albuginea. This structure is activated by means of the parasympathetic fibres originating from the sacral cord (ECKHARD 1863 GASKELL 1887 LANGELY & ANDERSON 1895). On the other hand it is known that in some animals erection is also induced by stimulating those fibres of the hypogastric nerve originating from the lumbar cord between the 2nd to 4th lumbar vertebrae (ECKHARD 1863 FRANÇOIS-FRANK 1895 BACQ 1935). It has been suggested that only the dilatation of the different-sized arteries is necessary for erection (ECKHARD 1863 KISS 1921). Moreover the constriction of the deep veins may be equally essential (DEYSACH 1939) As mentioned above, unstriated muscles are present in the walls of the cavernous sinusoids (HAM & LEESON 1961) which indicates that the erectile bodies themselves play an active role (NIKOLSKY 1879) Because of these findings the occurrence of different biologically active amines and that of acetylcholine in the mammalian penile erectile tissue have been investigated.

Methods

White laboratory rabbits weighing between 2.8–3.6 kg were used. The animals were at least six months old They were killed by a blow on the neck and by bleeding from the neck vessels. The penis was removed immediately and the corpus cavernosum urethrae and the corpora cavernosa penis were separated from each other and from the surrounding tissues The collagenous tunica albuginea was removed as completely as possible. All specimens were further treated immediately

The oxen also used as test animals were at least one year old and of the Ayrshire breed. The animals were killed in a slaughter house by perforating the frontal bone and bled by cutting the neck vessels. The specimens were dissected immediately after slaughtering and treated further within one hour. The tunica albuginea was removed completely from around the corpora cavernosa and glans penis. The urethral mucous membrane, which as a rule was easily separated, was removed from the corpus cavernosum urethrae and handled as a separate specimen.

Noradrenaline (NA) and adrenaline (A) were determined by the method of BERTLER *et al.* (1958): for purifying the extracts there was used a sodium-formed Dowex 50 X-4 ion-exchange resin from which NA and A had been eluted with N-HCl. The catecholamine content of the eluate was essentially determined fluorimetrically as described by v. EULER & LISHAJKO (1961) on the Aminco-Bowman spectrophotofluorimeter with activation wavelengths of 400 and 440 nm and fluorescence wavelengths of 510 and 540 nm, respectively (uncorrected instrumental values). The results were calculated by the procedure of VENDSALU (1960). The fluorescence characteristics of the extracts were similar to those of authenthic NA and A. By this method the recovery of NA was 89 % and that of A 83 %. None of the results presented here have been corrected for recovery.

The determination of dopamine (DA) was carried out by the method of BERTLER *et al.* (1958). From the ion-exchange resin NA and A were first eluted with N-HCl and then DA with 2 N HCl. In the latter fraction dopamine was determined spectrophotofluorimetrically by the method of CARLSSON & WALDECK (1958). The fluorescence characteristics were found to correspond with those of authenthic dopamine. The recovery by this method was 60 %.

To determine histamine the method of BARSOUM & GADDUM (1935), as modified by CODE (1937) was used. The histamine content of the purified extracts was estimated biologically on the guinea pig ileum. Mepyramine prevented the contractions induced by the test samples. The recovery was 71 %.

5-Hydroxytryptamine (5HT) was extracted by the acetone-heptane method (KÄRKI & PAASONEN 1959). The activities of the extracts were estimated biologically on a rat fundus preparation (VANE 1957). The recovery was 75 %, and methysergid (Deseril ®) prevented the contractions induced by the test samples.

Acetylcholine (ACh) contents of the tissue extracts were estimated on the frog rectus muscle preparation by GADDUM's superfusion method (1953) as modified by AHMED & TAYLOR (1957). This method is sensitive enough to estimate the extremely low ACh-concentrations. The Ringer solution used for superfusion included NaCl 0.65 %, KCl 0.014 %, $CaCl_2$ 0.012 %, $NaHCO_3$ 0.0 %. For the extraction of ACh, 3 ml of this solution (without $NaHCO_3$) were used, and the pH was adjusted to 4 with 0.01 N HCl. Physostigmine salicylate (10 μg/ml) was added to both superfusion and extraction solutions. Because the recovery of ACh is independent of whether the tissues are ground or cut to small pieces (ROTSCHUH 1954) the specimens were cut. The extracts were boiled on a hot water-bath for five minutes, centrifuged and neutralized, and the ACh-content was estimated immediately. Physostigmine (10 μg/ml) potentiated and d-tubocurarine (2 μg/ml) completely blocked the contractions induced by the test samples. They also lost their activity during the boiling in alkaline solution.

The drugs used: l-noradrenaline bitartrate and l-adrenaline (Orion Oy), 3-hydroxytyramine hydrochloride (Sigma Chemical Co.), 5-hydroxytryptamine creatinine sulphate (Fluka AG), acid histamine phosphate (British

Table I

Acetylcholine, histamine, 5-hydroxytryptamine, noradrenaline, adrenaline and dopamine contents, in µg/g ± SD of the penile erectile and urethral tissues of the ox. Numbers of specimens in parentheses.

Substance	Glans penis	Corpus cavernosum penis: Middle part	Corpus cavernosum penis: Proximal end	Corpus cavernosum urethra	Urethra
Acetylcholine	N activity (6)	No activity (6)	No activity (6)	0.39 ± 0.24 (10)	0.57 ± 0.27 (6)
Histamine	0.49 ± 0.25 (17)	0.49 ± 0.32 (15)	0.41 ± 0.30 (12)	0.81 ± 0.41 (9)	1.02 ± 0.43 (5)
5-Hydroxytryptamine	0.15 ± 0.07 (10)	0.11 ± 0.03 (9)	0.10 ± 0.04 (8)	0.16 ± 0.06 (7)	0.42 ± 0.20 (10)
Noradrenaline	0.01 ± 0.00 (4)	0.01 ± 0.01 (4)	0.01 ± 0.01 (4)	0.03 ± 0.03 (4)	0.03 ± 0.01 (4)
Adrenaline	0.02 ± 0.01 (4)	0.01 ± 0.00 (4)	0.03 ± 0.03 (4)	0.03 ± 0.01 (4)	0.06 ± 0.00 (4)
Dopamine	0.44 ± 0.02 (6)	0.36 ± 0.04 (6)	0.50 ± 0.11 (7)	0.32 ± 0.12 (7)	0.59 ± 0.08 (6)

Drug Houses, Ltd.) acetylcholine chloride (F Hoffmann-LaRoche & Co) physostigmine salicylate (C. H Boehringer Sohn) Deseril ® (Sandoz AG) scopolamine hydrochloride (C. H Boehringer Sohn) mepyramine maleate (Star Oy)

Results

The acetylcholine, histamine, 5-hydroxytryptamine, noradrenaline, adrenaline and dopamine contents of the penile erectile tissues of ox and rabbit are given in tables 1 and 2. Table 1 also gives the corresponding concentrations of the specimens taken from the urethral mucous membrane of the ox (*pars spongiosum urethrae*) It has not been possible to demostrate the presence of dopamine paperchromatographically in the penile erectile tissue of the rabbit.

Table 2

Acetylcholine, histamine, 5-hydroxytryptamine, noradrenaline and adrenaline contents, in μg/g ± SD of the penile erectile tissue of the rabbit Numbers of specimens in parenthesis.

Substance	Corpus cavernosum penis	Corpus cavernosum *urethrae*
Acetylcholine	0.47 ± 0.24 (8)	2.33 ± 0.80 (7)
Histamine	0 86 ± 0.28 (12)	4.39 ± 2.15 (6)
5-Hydroxytryptamine	0.22 ± 0 15 (9)	0.73 ± 0.53 (7)
Noradrenaline	0 28 ± 0 05 (8)	1 15 ± 0.35 (6)
Adrenaline	0.04 ± 0 02 (8)	0.17 ± 0 13 (6)

Discussion

By stimulating the parasympathetic fibres originating from the sacral part of the spinal cord, BACQ (1935) was able to show that the volume of the erectile tissue of a dog grows This reaction was potentiated by eserine and inhibited by atropine On the other hand HENDERSON & ROEPKE (1933) could not prevent increase in penile volume by means of atropine administered during the nerve stimulation. According to these investigators, the stimulation of the dilatory nerve fibres of the penis released locally a tissue hormone, whose action is potentiated by eserine but is not unequivocally abolished by atropine The discrepancy in the action of acetylcholine is to be explained first by DALE's (1914) observations that large quantities of acetylcholine cause erection, which is

inhibited by atropine, and secondly by the failure of HENDERSON & ROEPKE and BACQ to find acetylcholine in the perfusates of the test animal's erectile tissue. The mediator function of acetylcholine is also questionable, because it could not be found in the erectile tissue of the ox. However this observation can also be interpreted to mean that there is no active function in the erectile tissue, but that it is only a blood depot with a passive role. This is contradicted by the observation that in the walls of the cavernous sinusoids of the penis of some mammals there can be histologically shown to exist smooth muscle, which is innervated by the autonomic system and during erection relaxes together with the muscles of the walls of the helicinous arteries (HAM & LEESON 1961).

The role of histamine as a substance with a potent effect on smooth muscle is interesting. According to v. EULER & ÅSTROM (1948), stimulation of the postganglionic sympathetic fibre releases histamine: they assumed that peripherial organs could be stimulated in this way by irritating certain nerve fibres. In estimating the local significance of histamine, it must be remembered that the histamine content established in this study is low compared with that found by v. EULER & PURKHOLD (1951), for instance, in different tissues of the cow. According to them, it is also probable, taking into account the high histamine content of some nerve fibres, that at least a part of the histamine found in different peripheral organs is derived from these postganglionic sympathetic fibres.

As to 5-hydroxytryptamine content, the penis is an organ in which the concentrations are quite small. This substance has hardly any active role in penile erectile tissue.

The predominant catecholamine in the ox is dopamine, as is usual in ruminants (SCHÜMANN 1958, BERTLER & ROSENGREN 1959). As to the vasoactive effects of dopamine, it is known that in guinea pigs and rabbits it lowers the blood pressure (HOLTZ & CREDNER 1947) and that in dogs it is a renal vasodilatator (MCNAY *et al.* 1963 a). On the other hand in the last named test animal it decreases femoral blood flow (MCNAY *et al.* 1963 b). In rabbits the *proportion of adrenaline in the* total catecholamines is minimal, as is usual in peripheral organs.

Summary

The occurrence of the catecholamines, 5-hydroxytryptamine, histamine and acetylcholine in the penile erectile tissue and urethral mucous membrane of the ox and rabbit has been investigated. In the corpus cavernosum penis of the ox no ACh and rather small amounts of histamine and 5HT were found. The predominant catecholamine was dopamine.

ACh histamine and 5HT were also found there, the amounts being higher than in the ox. The possible role of these biologically active substances in the erection mechanism has been briefly discussed

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Hydroaminacrine III
Effect on Morphine-Induced Respiratory Depression in the Cat

By

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Previously the effects of hydroaminacrine on the actions of some depolarizing neuromuscular blocking agents have been investigated (DYRBERG *et al* 1962a & 1963) Hydroaminacrine is also known to have some respiratory effects. In animal experiments, hydroaminacrine has been shown to stimulate respiration that has been depressed by morphine (SHAW 1960) In experimental investigations on normal human subjects, hydroaminacrine has been found to have variable stimulatory effect on respiration (DYRBERG *et al* 1962b) On account of its respiratory effects, hydroaminacrine has been recommended for use on patients with respiratory depression after anaesthesia and for routine use along with narcotic analgesics to minimize the respiratory depressant effect of these agents (STONE *et al* 1961)

Our experiments were carried out mainly to compare the efficiency of hydroaminacrine with that of nalorphine, a specific narcotic antagonist, on morphine-induced respiratory depression.

Technique

Cats were anaesthetized with a mixture of chloralose (46 mg/kg body weight) and urethane (460 mg/kg body weight). After tracheotomy the respiratory rate and volume were continuously recorded, the latter by means of a Gildemeister gas meter (1922). The femoral vein was cannulated for injections.

Results

The results of the individual experiments are shown in table 1 Morphine (0.625–1 mg/kg i v) caused an average reduction in respiratory

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Table 1

Respiratory minute volumes and rates as percentages of initial values. Hydroaminacrine was administered 15 minutes after morphine, and nalorphine 1 hour after hydroaminacrine.

	Cat no.	Morphine 0.625–1 mg/kg	Hydroaminacrine 1.0 mg/kg	Nalorphine 0.3 mg/kg
volume	1	54	54	97
	2	34	39	83
	3	51	79	123
	4	62	66	91
rate	1	68	68	77
	2	67	100	91
	3	58	71	111
	4	72	69	95

volume to about 50 % of the initial values and of respiratory rate to about 66 %. After subsequent administration of hydroaminacrine (1 mg/kg i v given 15 minutes after administration of morphine) respiratory volume was practically unchanged in three of the animals, but considerably increased in one (from 51 to 79 % of the initial value). In this animal, respiratory rate was similarly increased (from 58 to 71 % of the initial value). In one of the other animals respiratory rate was also increased, but in the remaining two animals it was practically unchanged.

When the effect of hydroaminacrine was wearing off, about one hour later nalorphine (0.3 mg/kg) was injected, and now respiratory minute volume was in each case restored to near normal value, averaging 99 % of the initial value. Respiratory rates were restored to on an average, 94 % of the initial values.

Hydroaminacrine (1 mg/kg) was given to two cats that had not been pretreated and produced an increase in respiratory minute volume of 16 and 22 %, with an increase in rate of 11 % in one cat and no change in the other

Discussion

The claim that hydroaminacrine is a reliable stimulator of respiration gets little support from our experiments. Only in one of the four cats was alveolar ventilation improved when respiration had been depressed by the action of morphine, in contrast with the consistent and practically normalizing effect of nalorphine in similar circumstances. Respiratory rate was increased by hydroaminacrine in two cats, but an increased respiratory

rate has an adverse effect on alveolar gas exchange when unaccompanied by an increase in respiratory minute volume Further the effect of hydroaminacrine on respiration is of relatively short duration compared with that of nalorphine, which has been found to last several hours (Hougs, unpublished results)

The finding that respiration is stimulated when hydroaminacrine is given alone points to this drug's being more of an analeptic than a specific narcotic antagonist The effect of nalorphine given alone is respiratory depression

In cats as in man (DYRBERG *et al* 1962b) hydroaminacrine appears to be an unreliable antagonist of opiate induced respiratory depression.

Summary

When given after a dose of morphine, which depressed respiratory volume to about 50 %, hydroaminacrine improved alveolar ventilation somewhat in one of four cats The effect of nalorphine on the same cats was consistently to restore the respiratory volume to near normal. When given without pretreatment with morphine, hydroaminacrine increased respiratory volume moderately

The experiments indicate that the stimulating effect of hydroaminacrine on morphine-depressed respiration is unreliable, in contrast with the respiratory effect of nalorphine under the same conditions.

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The Effect of Drugs on the Duration of Toxic Symptoms Caused by Sublethal Doses of Local Anaesthetics

An Experimental Study on Mice

By

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Recent investigations have shown that many drugs administered before other drugs may affect both the duration and the efficacy of the latter's action. This has often been found to be the result of an inhibitory or stimulatory effect of the first compound on the activity of the drug metabolizing enzymes in the microsomes of the liver (CONNEY & BURNS 1962). The action is directed especially at enzymes oxidatively metabolizing various drugs and requiring the presence of reduced triphosphopyridine nucleotide (TPNH) and oxygen for this process (REMMER 1962). Some drugs, however have also been found to affect the activity of other kinds of microsomal enzymes. Thus, for example, phenobarbital stimulates the procaine-hydrolyzing "procainesterase" in the microsomes of the rat liver (REMMER 1962, 1963) and iproniazid, on the other hand, acts as an inhibitor of this "procainesterase" (FOUTS & BRODIE 1956).

Since the toxic action of a local anaesthetic is affected in part by its rate of metabolism, it seemed justifiable to study the effect of various drugs on the duration of toxic symptoms caused by local anaesthetics. Three representatives of the aminoacylamide group were chosen for study i. e. lidocaine, mepivacaine and citanest ® (a-n-propylamino-2 methylpropionanilide). It is known that lidocaine is oxidatively metabolized by a microsomal enzyme system that requires the presence of TPNH and oxygen (HOLLUNGER 1960).

Methods

Male albino mice weighing 20–24 g were used. The test room temperature was 24 C. The rectal temperatures of the mice were measured with thermocouples. No significant temperature differences were observed between the premedicated and the control mice.

A comparison was first made of the duration of the toxic state after a sublethal dose of lidocaine (175 mg/kg s.c.) in mice that had been premedicated with various drugs and in control mice. The dose of lidocaine chosen was sufficient to cause loss of the righting reflex within a few minutes and to produce a state similar to general anaesthesia (FRANK & SANDERS 1963) with respiration maintained at a satisfactory level. Before as well as after the narcosis proper there was motor restlessness and weakness and also convulsions. The duration of the toxic state was defined as the time between the injection of the anaesthetic and the return of the righting reflex. The latter was regarded as positive when the animal was able to stand up and after being laid successively on either flanks was able to get up again.

The inhibitory action of drugs on the microsomal enzymes of the liver has generally been observed immediately after their administration, whereas a possible stimulatory action has manifested itself slowly reaching a maximum about 48 hours after administration of the drug (CONNEY & BURNS 1962) Hence in our study the drugs were administered generally 1 or 48 hours before the experiment.

The local anaesthetics used were lidocaine hydrochloride (xylocain ® Astra), mepivacaine hydrochloride (carbocain ® Bofors, Nobelkrut), citanest ® hydrochloride (Astra)

The drugs used in premedication were barbital sodium, phenobarbital sodium, pentobarbital sodium, thiopental sodium (intraval ® sodium May & Baker), nikethamide (corditon ® Medica) bemegride (megimide ® Nicholas) chlordiazepoxide (librium ® Hoffmann-LaRoche) chlorpromazine hydrochloride (largactil ® May & Baker) meprobamate, chloramphenicol sodiumsuccinate (chloramphen ® Boehringer & Soehne) iproniazid phosphate (Hoffmann-LaRoche) isoniazid (rimifon ® Hoffmann-LaRoche) and D L-ethionine (Mann Research Lab., New York).

The local anaesthetics and ethionine were dissolved in physiological saline and the other drugs in distilled water The volume injected was 0.1 ml/10 g, exception for meprobamate, which was given in a volume 0 4 ml/10 g.

The effects of phenobarbital premedication on the cumulative toxicity of lidocaine, mepivacaine and citanest were also studied Repeated intraperitoneal injections of each local anaesthetic were given to eight control mice and to eight mice that had received 90 mg/kg of phenobarbital 48 hours previously Each local anaesthetic was given 5 times, according to the regimes lidocaine, 40 mg/kg at 20 min. intervals, mepivacaine, 30 mg/kg at 15 min. intervals, and citanest 70 mg/kg at 15 min intervals. The volume of the individual doses was 0 1 ml/20 g.

Results and Discussion

Of the drugs given 48 hours before the experiment only phenobarbital and barbital shortened the duration of the toxic symptoms produced by lidocaine (table 1)

According to DORFMAN and GOLDBAUM (1947) about one-third of a dose of phenobarbital is decomposed in the mouse body in 24 hours even if a part is excreted unchanged in the urine, it may be assumed that minute amounts of the drug are still present in the mouse 48 hours after administration Such a comparatively small amount of phenobarbital might be assumed to decrease the toxic symptoms produced by

Table 1

The effect of different drugs on the duration of toxic symptoms caused by lidocaine. The drugs used for premedication were given intraperitoneally and lidocaine (175 mg/kg) subcutaneously. Each test group and each control group contained ten mice. The values given represent averages ± standard error. *The value of p is given* if the difference between the test group and corresponding control group is significant ($p < 0.05$).

Premedication	Dose mg/kg	Interval (hours)	Duration of toxic symptoms (min)		p
			premedicated	controls	
Phenobarbital	90	48	44.4 ± 2.7	62.3 ± 2.8	< 0.001
	45	48	48.0 ± 1.9	62.3 ± 2.8	< 0.001
	90 + 90	72 and 48	37.2 ± 3.1	55.1 ± 3.5	< 0.01
	20	3	60.8 ± 5.9	55.1 ± 3.5	
	5	3	53.3 ± 2.1	55.8 ± 4.5	
	1	3	71.6 ± 5.3	74.5 ± 6.1	
	0.1	3	67.0 ± 3.0	64.8 ± 3.0	
Ethionine	250	48	70.8 ± 4.5	71.5 ± 4.1	
Phenobarbital	90	48	55.6 ± 2.4	71.5 ± 4.1	< 0.01
Ethionine + Phenobarbital	250 / 90	48.5 / 48	67.8 ± 3.4	71.5 ± 4.1	
Barbital	125 + 125	72 and 48	45.5 ± 3.2	57.8 ± 4.2	< 0.05
Thiopental	30 + 30	72 and 48	52.2 ± 2.5	57.8 ± 4.2	
Pentobarbital	25 + 25	72 and 48	52.5 ± 4.4	55.1 ± 3.5	
Lidocaine	50 + 50	72 and 48	61.1 ± 4.7	57.8 ± 4.2	
Chlordiazepoxide	50 + 50	72 and 48	44.4 ± 4.6	51.2 ± 4.7	
Meprobamate	200	48	53.5 ± 2.9	55.5 ± 3.2	
Chlorpromazine	5	48	61.4 ± 2.9	66.2 ± 3.3	
Nikethamide	100	48	54.5 ± 2.6	55.5 ± 3.2	
Bemegride	10 + 10	72 and 48	51.2 + 2.8	51.2 ± 4.7	
Iproniazid	100	1	110.7 ± 9.4	58.2 ± 3.7	< 0.001
	100	4	103.9 ± 6.9	58.2 ± 3.7	< 0.001
	100	24	68.0 ± 6.1	58.2 ± 3.7	
	100	48	60.4 ± 3.7	63.1 ± 2.3	
Isoniazid	60	1	91.7 ± 5.7	62.8 ± 4.8	< 0.001
	60	48	61.8 ± 3.8	63.1 ± 2.3	
Chloramphenicol	100	1	95.9 ± 6.7	62.8 ± 4.8	< 0.001
	100	48	61.6 ± 2.6	66.2 ± 3.3	

local anaesthetics. However the fairly small amounts of phenobarbital administered a few hours before the experiment did not shorten the duration of the toxic state caused by lidocaine (table 1). Thus, in the course of 48 hours phenobarbital has apparently produced in the mouse organism changes that reduce the duration of the toxic symptoms caused by lidocaine.

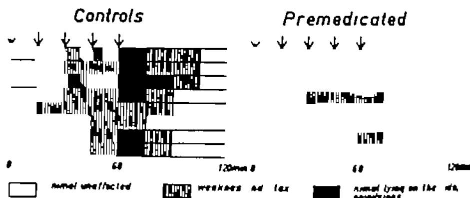


Fig. 1 Cumulative toxicity of citanest in eight control mice and in eight mice premedicated 48 hours earlier with phenobarbital (90 mg/kg). Each horizontal line represents the behaviour of one animal during and after repeated intraperitoneal injections of citanest Single doses (70 mg/kg) were given at 15 minutes intervals (arrows)

Ethionine is a strong inhibitor of protein synthesis, and its use has made possible inhibition of the stimulatory action of certain drugs on the enzymes of the liver microsomes. (CONNEY & BURNS 1962). The stimulation is believed to be based on an increased formation of enzymes. Ethionine itself did not affect the duration of the toxic state, but given half an hour before the administration of phenobarbital it was capable of inhibiting the shortening effect of phenobarbital on the duration of toxic symptoms caused by lidocaine (table 1) This seems to indicate the stimulation by phenobarbital of the enzyme system that metabolizes lidocaine

This assumption is also supported by the results obtained in the cumulative toxicity experiments The toxic symptoms after lidocaine, mepivacaine and citanest appeared at an earlier stage in the control mice than in mice given a premedication of phenobarbital The symptoms in the control group were also more severe and lasted longer Fig. 1 shows the effect of phenobarbital on the cumulative toxicity of citanest. Similar results were obtained with lidocaine and mepivacaine.

On the other hand, a number of other drugs did not affect the duration of toxic symptoms caused by lidocaine when they were given 48 hours before the experiment (table 1) However KATO *et al* (1964) among others, have observed that thiopental, pentobarbital chlorpromazine, nikethamide and meprobamate for example, have a stimulatory effect on some of the enzymes in the microsomes of the rat liver These substances are thus able to stimulate the metabolism of for instance, pentobarbital, hexobarbital, meprobamate and strychnine. The fact that these drugs did not affect the duration of the toxic state caused by lidocaine may be due to dissimilarities in their ability to stimulate various enzymes

(CONNEY & BURNS 1962). There may also be a species difference. The mouse metabolizes hexobarbital, for instance, considerably more rapidly than the rat (BRODIE 1962), and it is to be assumed that substances metabolized faster do not have time to stimulate the microsomal enzymes to the same extent as substances of a similar type, but with a slower rate of metabolic breakdown.

Iproniazid (LAROCHE & BRODIE 1960), isoniazid (LESSIN 1959) and chloramphenicol (DIXON & FOUTS 1962) inhibit the metabolism of numerous drugs, for instance, certain barbiturates. When these drugs were given 1 hour before the experiment the toxic state caused by lidocaine was clearly prolonged (table 1). The effect of iproniazid does not, however, show any correlation with its inhibitory effect on monoamine oxidase (LAROCHE & BRODIE 1960). Investigations to be carried out later may possibly shed light on the question whether the prolongation of lidocaine narcosis by these drugs is due to a slowing down of the rate of its metabolism.

Summary

Phenobarbital and barbital administered 48 hours previously shorten the duration of toxic symptoms caused in mice by lidocaine, whereas iproniazid, isoniazid and chloramphenicol given 1 hour previously prolong the symptoms. Ethionine is capable of inhibiting the shortening effect of phenobarbital on the duration of the toxic state caused by lidocaine. Phenobarbital administered 48 hours previously clearly reduces the cumulative toxicity of lidocaine, mepivacaine and citanest. From the results it is concluded that there may be an inhibitory or stimulatory effect of certain drugs on the metabolism of these local anaesthetics.

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The Effect of Adrenaline on the Toxicities and Absorptions of L 67 (Citanest ®) and Some Other Local Anaesthetics studied in Mice and Rabbits

By

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The addition of a vasoconstrictor to solutions of local anaesthetics is known not only to increase their efficiency but also to decrease their toxicity in clinical use. As a result of vasoconstriction at the site of injection a high concentration of the agent is maintained for a longer period of time in the nerve structure, thus prolonging the duration of anaesthesia and also at certain injection sites increasing the incidence of satisfactory anaesthesia. Since the rate at which the local anaesthetic agents enter the systemic circulation is reduced by the local vasoconstriction, the systemic toxicity is decreased.

Pharmacological studies have repeatedly confirmed the observation first made by BRAUN (1903) that the activity of local anaesthetic agents is increased by a vasoconstrictor such as adrenaline. On the other hand, the vasoconstrictors have only exceptionally been found to decrease the toxicity of these agents in laboratory animals. This fact has recently been discussed by AVANT & WEATHERBY (1960), who found that only with tetracaine did the addition of adrenaline produce a highly significant reduction in systemic subcutaneous toxicity in mice.

The purpose of our investigation has been to study the effect of adrenaline on the acute toxicity of a new local anaesthetic agent, o-methyl-α-propylaminopropionanilide, (L 67 Citanest ®), which was synthesized by LÖFGREN & TEGNÉR (1960). This has been compared with lidocaine, procaine and tetracaine. Acute subcutaneous and intravenous toxicities have been determined in mice, and the rates of absorption from different injection sites have been studied in mice and rabbits.

Methods

Acute subcutaneous toxicity was determined on albino inbred mice (DSS, 17 22 g) fasted for 24 hours before the test. The solutions of each agent alone and in the presence of adrenaline were studied on the same day on the same batch of animals kept under identical conditions. At least four different dose levels were used for each agent alone and along with different amounts of adrenaline. During the observation period (24 hours) 10 animals were kept in the same cage. The LD50 values were calculated by the graphical method of MILLER & TAINTER (1944). The significance of the differences in toxicity observed between the agents alone and with adrenaline was calculated by Student s t test.

Acute intravenous toxicity was determined on the same strain of mice, kept and observed under the same conditions as those for the subcutaneous series. Tetracaine was used in 0.025 %, lidocaine, L 67 and procaine in 0.25 solution. Injections were made in the tail vein at about 0.1 ml/second. Adrenaline was always used at a concentration of 1 400000 The results were statistically analysed by the same method as for the subcutaneous series.

Determinations of the plasma concentrations of lidocaine and L 67 in mice and rabbits were made by the method of WOODS, COCHIN FORNEFELD MCMAHON & SEEVERS (1951). The blood sample was centrifuged, and to 5 ml plasma 6 ml toluene and 2 ml 2 N NaOH were added. The mixture was shaken for 20 min. After centrifugation 4 ml of the toluene phase were transferred to a separating funnel and extracted twice with 2 ml 1 N HCl and once with 1 ml water The aqueous phase was transferred to a second separating funnel containing 4 ml toluene and 3 ml 2 N NaOH and shaken for 10 min. To the toluene phase was added 0.4 ml bromocresol purple solution (25 mg bromocresol purple dissolved in 50 ml toluene), and the optical density of the solution was measured at 420 mμ. A standard curve was made with identically treated plasma samples to which known amounts of the local anaesthetic agent had been added. The agents were given subcutaneously to mice, 100 mg/kg as 1 solution, and the adrenaline concentrations were 1 200000 and 1 80000 The blood obtained from five animals at 15 min. was pooled to yield a sufficient quantity for the analysis. Four groups of 5 animals were used for each agent. In the rabbits (2.0–3 0 kg) blood was obtained by heart puncture performed twice in each rabbit, once before and again at one of several time intervals, up to 2 hours after the injection of the local anaesthetic agent. Injections were made either subcutaneously or submucosally into the buccal fold in the oral cavity as described by HARNISCH (1953) By both routes the dose was 20 mg/kg of the local anaesthetic agent (2 solution), and the adrenaline was added at concentrations of 1 80000 or 1 200000.

All local anaesthetic agents were dissolved as hydrochlorides, and the pH was adjusted to 6.5 All toxicity figures are for the local anaesthetics as hydrochlorides. Adrenaline was used as bitartrate, but the doses given refer to the free base.

Results

Acute subcutaneous toxicity in mice For each local anaesthetic solution the toxicity both without and with different amounts of added adrenaline was determined under identical conditions. The results are shown in table I In order to keep the *injected volumes*, and hence the amounts of adrenaline, as uniform as possible, it was necessary to use

Table 1

Effect of adrenaline on the acute subcutaneous toxicity to mice of some local anaesthetics (LA).

LA agent	No of animals	Adrenaline concentration ×1000⁻	LD50 ± s.e.m. LA g/kg	Significance of difference p	Adrenaline at LD50 mg/kg *)
Procaine 4%	60	0	0.42 ± 0.03		0
	50	1 400	0.30 ± 0.02	$p < 0.001$	0.02
	60	1 200	0.36 ± 0.02	$p > 0.05$	0.05
	60	1 100	0.36 ± 0.02	$p > 0.05$	0.09
	50	1 50	0.36 ± 0.02	$p > 0.05$	0.18
Lidocaine 2%	120	0	0.34 ± 0.02		0
	120	1 400	0.20 ± 0.01	$p < 0.001$	0.03
	120	1 200	0.20 ± 0.01	$p < 0.001$	0.05
	120	1 100	0.23 ± 0.01	$p < 0.001$	0.11
L 67 4%	60	0	0.60 ± 0.05		0
	50	1 400	0.45 ± 0.02	$0.01 > p > 0.001$	0.03
	60	1 200	0.43 ± 0.02	$0.01 > p > 0.001$	0.05
	60	1 100	0.41 ± 0.03	$p < 0.001$	0.10
	50	1 50	0.38 ± 0.02	$p < 0.001$	0.19
Tetracaine 0.5%	60	0	0.03 ± 0.002		0
	60	1 400	0.06 ± 0.004	$p < 0.001$	0.03
	50	1 200	0.07 ± 0.003	$p < 0.001$	0.07
	40	1 100	0.06 ± 0.003	$p < 0.001$	0.12
	60	1 50	0.07 ± 0.004	$p < 0.001$	0.26

*) The LD50 of adrenaline in this series of mice was 4.6–5.8 mg/kg, as determined on 5 groups of 60–90 animals.

different concentrations of the local anaesthetics. The less toxic agents L 67 and procaine were used in 4 %, lidocaine in 2 % and tetracaine in 0.5 % solutions. In the table the amounts of adrenaline in the LD50 dose are given separately

Only with tetracaine did the added adrenaline reduce toxicity at all concentrations studied. For all the other agents the toxicity tended to be increased by the added adrenaline. With L 67 the increase in subcutaneous toxicity became statistically highly significant only at the highest adrenaline concentrations. With increasing adrenaline concentrations, from 1 400000 to 1 50000, there was no progressive increase in toxicity of the procaine and lidocaine solutions. Similarly the reduction in tetracaine toxicity was little influenced by an eightfold increase in adrenaline concentration.

The figures in table 1 for the toxicity of lidocaine and L 67 alone are in good agreement with those previously obtained by WIEDLING (1960). However it should be noted that the experiments were performed in

such a way that direct comparisons of the results presented in table 1 can be made only between each agent by itself and with different amounts of adrenaline For the different local anaesthetic agents different batches of mice were used at time intervals of one week or more. Thus, although the LD50 values in table 1 indicate that L 67 alone was less toxic than procaine alone, it is not legitimate to conclude that this is generally true. Direct comparisons of these agents usually show that procaine is somewhat less toxic than L 67 in a separate study made in our laboratories conforming to the recommendations given in the *Pharmacopoeia Nordica* (1963) we found the following LD50 values for procaine 0 8 ± 0.04 for L 67 0 6 ± 0 06 g/kg.

Onset of toxic symptoms On subcutaneous injection of lidocaine into mice toxic symptoms appeared after a shorter latency period than occurred with L 67 A special study of this difference was made on groups of 12 mice, which received LD50 doses of the two agents without adrenaline under the same conditions as in the tests summarised in table 1 Convulsions were observed in all animals. With L 67 they occurred after 4 4 ± 0 4 min. and with lidocaine after 1 9 ± 0 1 min. Loss of the righting reflex was observed after 6 6 ± 0 5 (freq 11/12) and 2.5 ± 0.1 (12/12) min. respectively The addition of adrenaline, 1 200000, roughly doubled the latency time for toxic symptoms from lidocaine, whereas with L 67 the latency time increased only by about 20–25 %.

The effect of adrenaline on the development of toxic symptoms after subcutaneous lidocaine injection was also studied in a small series of mice in which the heart rate was continuously recorded (by ECG) from the time when the animals became sedated Five animals received 400 mg/kg lidocaine (2 % solution) subcutaneously and another 5 animals the same dose with adrenaline 1 200000 (0 1 mg/kg) Of the group of 5 animals receiving lidocaine alone, 4 died within 38 min. and their heart rates fell from an average of 400–500 beats/min at 10 min to less than 100 beats/min. within 15–22 min Of the group of five receiving lidocaine plus adrenaline 4 were still alive after 90 min The average heart rate of these animals was 500 beats/min at 25 min and decreased only slowly to about 400 beats/min at 60–70 min Although this was not evidenced by the LD50 determinations (table 1) these results indicate that adrenaline retarded the rate of absorption of lidocaine

Acute intravenous toxicity in mice The volumes ordinarily injected in intravenous toxicity studies on mice are of the order of 0.2–0.5 ml If a high concentration of adrenaline is used along with the local anaesthetic solution, the amount of adrenaline itself may therefore be large enough to produce toxic effects or even deaths. Throughout our study a low concentration of adrenaline 1 400000 was therefore used. The

Table 2.

Effect of adrenaline (1 400000) on the acute *intravenous* toxicity to mice of some local anaesthetics (LA).

LA	No of animals	Adrenaline concentration ×1000	LD50 ± s.e.m. LA mg/kg	Significance of difference P	Adrenaline at LD50 mg/kg
Procaine 0.25%	70 70	 1 400	47.5 ± 3.3 33.8 ± 2.8	0.01 > p > 0.001	0.03
Tetracaine 0.25%	80 50	 1 400	5.6 ± 0.31 5.1 ± 0.16	p > 0.05	0.02
Lidocaine 0.25%	40 60	 1 400	25.0 ± 1.6 20.8 ± 1.5	p > 0.05	– 0.02
L 67 0.25%	50 50	 1 400	45.0 ± 2.5 37.5 ± 2.5	0.05 > p > 0.01	0.04

The L LD50 of adrenaline in this series of mice was 0.18–0.24 mg/kg as determined on 3 groups of 50–70 animals.

dose of adrenaline thus ranged between 0.02–0.04 mg/kg, which was 1/5–1/10 of the i.v. LD50 dose for our strain of mice.

As shown in table 2, all the local anaesthetic solutions tested tended to become more toxic when adrenaline was added. The toxicities found under the conditions applied in this study are of the order of magnitude to be expected if the toxicity of each individual local anaesthetic agent is added to that of adrenaline.

Plasma concentrations in mice Plasma analyses were made after subcutaneous injections of lidocaine and L 67 alone or along with adrenaline, a high dose of the local anaesthetic agent being used so as to ensure that toxic reactions would be produced. The blood was collected at a time when toxic reactions were commonly observed (after 15 min.). With lidocaine plus adrenaline, the concentration in the plasma at 15

Table 3

Concentrations of local anaesthetics (LA) in µg/ml (base) found in the plasma 15 minutes after subcutaneous injection of 100 mg/kg into mice.
= number of animals.

LA	Adrenaline concentration		
	zer	1 200000	1 100000
Lidocaine	15 4 0.7 (4 5)	11.2 ± 1.0 (= 6 5)	12.1 ± 0.7 (n = 4 × 5)
L 67	7.0 ± 0 6 (= 4 5)	6.5 ± 0.3 (= 4 5)	

Table 4

Concentrations of local anaesthetics (LA) in µg/ml (base) found in plasma at different times after subcutaneous injection of 20 mg/kg into rabbits.

LA	Time after injection min	Concentration of adrenaline zero	1 200000	1 80000
Lidocaine	10	4.5 6.2, 6 7	1 1 1.3	0.6, 0.8
	20	4 8 5 8 6.8	1 7 1 8	1.2, .2
	30	3 5 3 9 4 9	1.8 2.0	1 6, 3 0
	60	1.5 1 7 2.8	1 4 3 2	1.8, .4
	90	0.7 0 9	1 2, 1 7	1., .0
	120	0.5 0 7	0 7 2.0	0.9 1.5
L 67	10	0.7 0 9 1 4	0 9 1 5	0 4 0 4
	20	1 0, 1 4 2.3	1 1 2.2	0.4 0.6
	30	0.9 1 3 2.0	1 6 1 9	0.6, 1 2
	60	0.4 0 6, 1 2	1 1 1 6	0.7 1.3
	90	0 4 0.5 0.7	0.5 0.7	0.8, 0.9
	120	0.2, 0 5 0.6	0 3 0.5	0.6, 0.8

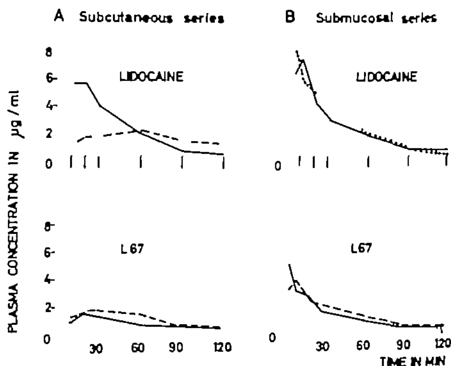


Fig. 1 Rabbits. Plasma concentrations of lidocaine and L 67 alone or along w th adrenaline, at different times after s bcutaneous (A) or I tra-oral submucosal (B) injections of ^0 mg/kg.

Means of figures in tables 4 and 5
——— lidocaine or L 67 without adrenaline.
– – – – adrenaline 1 ^00000.
adrenaline 1 80000.

Table 5

Concentrations of local anaesthetics (LA) in µg/ml (base) found in plasma at different times after intra-oral submucosal i jection of 20 mg/kg into rabbits.

LA	Time after injection min.	Concentration of adrenaline: zero	1 200000	1 80000
Lidocaine 2%	5	5.5 7.2		7.3 8.7 8.9
	10	6.9 8.4		5 7 6.4
	20	3 9 4.8		4.7 5 4
	30	2.8, 3.8		4.2, 4.9
	60	1 6, 2.4		1.5 2.7
	90	0.9 1.5		0 8, 1 0
	120	0.7 1 1		0.4 0.6
L 67 2%	5	5.2, 5.3	3.2, 3.4	
	10	2.7 3.6	2.8, 3.7 5.0	
	20	2.6, [illegible]	2.2, 2.7	
	30	1.5 1 7	1.8, 2.0	
	60	0.7 1.0	1.2, 1.3	
	90	0 3, 0.4	0.4, 0 6	
	120	0.2, 0.3	0.2, 0.4	

min. was somewhat lower than that observed with lidocaine alone ($0.01 > p > 0.001$) As shown in table 3 the L 67 concentration, however was not affected to a significant degree. It should be noted that an increase in adrenaline concentration from 1 200000 to 1 80000 did not cause any further reduction in the plasma concentration of lidocaine at 15 minutes. The table also shows that for lidocaine the plasma concentrations are about twice as high as for L 67 at 15 min., which would seem to indicate a slower rate of absorption of L 67 whether alone or in the presence of adrenaline.

Plasma concentrations in rabbits.

A. *Subcutaneous series* Solutions of lidocaine and L 67 (20 mg/kg of solutions), with or without adrenaline, were injected subcutaneously into the backs of rabbits. At least 2 rabbits were used for each test, at time intervals of 10, 20, 30 60, 90 and 120 min. (total, 106 animals).

In the absence of adrenaline the highest plasma concentrations were obtained with lidocaine and the lowest with L 67 (table 4). These results are in general agreement with those obtained on mice (table 3) The addition of adrenaline, 1 200000, clearly reduced the plasma concentration of lidocaine. Here also an increase in adrenaline concentration from 1 200000 to 1 80000 caused no further reduction in plasma concentration of lidocaine. The mean values of the concentrations from table 4 are shown graphically in fig. 1 A.

B *Submucosal series* The serum concentrations of the same local anaesthetic solutions were also studied after intra-oral submucosal injection into the buccal fold The same experimental procedure as in the subcutaneous series was applied 20 mg/kg of each agent being injected in 2 / solutions The results are given in table 5 The mean values of the concentrations found are shown graphically in fig. 1 B Contrary to the results obtained in the subcutaneous series, the addition of the vasoconstrictor at the concentrations studied did not significantly reduce the plasma concentrations of the local anaesthetics. Here also the serum concentrations of L 67 whether alone or in the presence of adrenaline, were clearly lower than those of lidocaine.

Discussion

The results obtained in our investigation have shown that when different local anaesthetic agents were injected subcutaneously into mice, only tetracaine became less toxic if given along with adrenaline at concentrations ranging between 1 400000 and 1 20000. For the other agents studied viz. procaine, lidocaine and L 67 the addition of adrenaline either produced no change or caused an increase in toxicity These results are in good general agreement with those obtained by HOLLER (1952) for lidocaine and procaine and with those found by AVANT & WEATHERBY (1960) also for tetracaine

A comparison between L 67 and lidocaine in equally toxic doses in the absence of adrenaline showed that, after subcutaneous injection into mice, the toxic symptoms developed more slowly from L 67 This would seem to indicate that L 67 is absorbed from the injection site at a slower rate than lidocaine. The analysis of the plasma concentrations of the two agents after 15 min (table 3) supports this conclusion This difference in rate of absorption of the local anaesthetics as such would seem to be the first factor to be considered when trying to explain the effect of an added vasoconstrictor upon the toxicity of various local anaesthetic solutions.

The addition of adrenaline did not reduce the toxicity of lidocaine and L 67 when these agents were injected subcutaneously into mice. With lidocaine, however adrenaline delayed the onset of toxic symptoms, and the plasma concentration at 15 min was lowered The fact that this effect of adrenaline did not reduce lidocaine toxicity may at least in part, be due to a relatively slow rate of detoxification of this compound. Addition of adrenaline to L 67 solutions did not delay the onset of toxic symptoms to a similar degree, nor did it significantly affect the plasma

concentration 15 min. after the subcutaneous injection. This difference between lidocaine and L 67 may be largely due to the difference in absorption rate of the local anaesthetics as such. Lidocaine, being more rapidly absorbed, would be expected to be more affected by the vasoconstrictor than the more slowly absorbed L 67. However the difference may also be in part due to various degrees of antagonism of the local anaesthetic agents against the vasoconstriction produced by adrenaline. It has been shown on isolated strips of rabbit aorta that the contraction produced by adrenaline is counteracted more by L 67 than by lidocaine (ÅSTRÖM 1964).

When discussing the role of adrenaline in the toxicity of local anaesthetic solutions it would seem pertinent to consider its local as well as its systemic effects. The systemic effects of absorbed adrenaline include an enhancement of systemic circulation, and this in turn could tend to cause a more rapid distribution of the agents, for example to the brain. The systemic effects of adrenaline would thus tend to counteract the retardation of absorption caused by the local action of adrenaline. The systemic effects would seem to be a factor of particular importance in small animals like mice, in which the total amount of adrenaline per kg body weight becomes relatively high when local anaesthetic solutions with clinically used adrenaline concentrations are injected. The observation that an eightfold increase in adrenaline concentration caused practically *no change in the LD50 values in mice (table 1) may be explicable* by this dual action of adrenaline. The significance of the size of the experimental animals has indeed been indicated in some previous studies. Thus LUDUENA, HOPPE, COULSTON & DROBECK (1960) found that the addition of L-nordefrin (Cobefrine ®), 1:20000, increased the toxicity of mepivacaine when injected subcutaneously into mice, but reduced the toxicity by the same route in guinea pigs and rabbits.

The balance between the local and the systemic effects of local anaesthetic solutions containing adrenaline would be expected to vary with the type of tissue injected. The significance of this factor was emphasized by the results obtained from plasma analysis in rabbits receiving the injections submucosally in the oral cavity. Under the conditions of these experiments, adrenaline did not retard the absorption of lidocaine at all. This result may be compared with that obtained in dental infiltration anaesthesia in man, when adrenaline has been found to delay the absorption, as indicated by the increase in anaesthetic effect (e. g. Huldt 1953).

The toxicity of solutions of local anaesthetics containing adrenaline thus seems to depend upon several factors: the rate of absorption of the local anaesthetic itself, the effect of the agent *on the local vasoconstrictor*

action of adrenaline and the balance between local and systemic effects of adrenaline The latter effects are in turn greatly influenced by the experimental conditions the amount of adrenaline given, the size of the animal and the properties (vascularization) of the tissue into which the injection is made. Finally the toxicity of a local anaesthetic, whether with or without adrenaline will be affected by its pattern of distribution and rate of elimination (metabolism) in the organism. Major differences in these properties have been demonstrated between L 67 and lidocaine (ÅSTRÖM, PERSSON and ROSS, unpublished results)

Because of the complex interaction between these various factors in different experimental situations, it is difficult to find a pharmacological model of clinical relevance for the testing of the toxicity of local anaesthetic solutions containing adrenaline The results obtained in this investigation indicate that LD50 values derived from toxicity studies in mice have only a limited value for the prediction of toxicity to man. The use of experimental animals larger than mice would seem to offer certain advantages, but when transferring the results to the conditions in man great caution must be exerted, and due consideration must be given to the difference in scale factors

Our investigation was undertaken with the primary purpose of studying the effect of adrenaline on the toxicity of L 67 compared with that of lidocaine In the different tests conducted adrenaline had the same qualitative effect on L 67 as on lidocaine, but quantitatively its action on L 67 was less pronounced From the results obtained it may also be concluded that, since the effect of adrenaline on absorption seems to vary with the local anaesthetic agent, the optimum concentration of the vasoconstrictor for its toxicity and probably also for its action, may be expected to vary with the type of anaesthetic agent used.

Summary

The toxicity of some local anaesthetic solutions alone and in the presence of adrenaline was studied after subcutaneous or intravenous administration to mice and the rates of their absorption by mice and rabbits were determined.

The lower toxicity of L 67 than of lidocaine was confirmed. When injected subcutaneously into mice, adrenaline increased the toxicity of L 67 in a manner similar to its enhancement of lidocaine toxicity In these experiments only tetracaine toxicity was reduced by adrenaline.

On the basis of absorption studies, it is concluded that the action of adrenaline in local anaesthetic solutions depends upon several factors,

such as the rate of absorption of the local anaesthetic itself the modifying effect of the anaesthetic on the local vasoconstrictor action of adrenaline and the balance between the local and the systemic effects of adrenaline. The latter in turn, depends on the experimental conditions, e. g. the total amount of adrenaline given, the size of the animal and the type of tissue injected.

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The Oxidation and Bromination of some Phenothiazine Derivatives

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In 1953 DUBOST & PASCAL reported the formation of a red solution when concentrated sulphuric acid was mixed with a solution of largactil ® (chlorpromazine) This reaction was used by them to determine colorimetrically the amount of the tranquillizer in biological fluids. When using this reaction in a study of the excretion of fluopromazine (vesprin ®), STREET (1962a) observed that the coloured solution formed by chlorpromazine in 50% sulphuric acid exhibited characteristic ultraviolet absorption curves He tabulated the ultraviolet absorption curves for 6 phenothiazine derivatives

In three excellent articles, BORG & COTZIAS (1962, a b and c) have reported their studies of the interaction of trace metals with phenothiazine derivatives. They demonstrated that chlorpromazine and several other phenothiazine derivatives gave rise to coloured products on treatment with any of the trivalent cations of iron cobalt and manganese. For a given derivative, each of the cations yielded the same product They suggested that the divalent sulphur in the phenothiazine molecule was essential for colour formation, because no colour was produced when these cations were added to the sulphoxide of the phenothiazine derivative

Using absorption spectrometry oxidimetric titration, electrolysis and electron spin resonance spectrometry these workers claimed to have identified the chromophore formed from phenothiazine derivatives by the action of the cations Fe^{3+} Co^{3+} Mn^{3+} Ce^{4+} or the persulphate anion S_2O_8 as a semiquinone free- radical ion They suggested that further oxidation, involving the loss of a second electron would yield a

bivalently oxidised quinonoid form" (I) and that this phenazothionium ion would react with water to form the sulphoxide (II) according to the equation in fig. 1

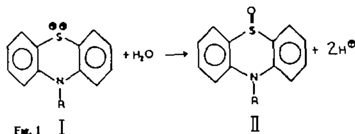


Fig. 1

It was suggested that under the mild conditions employed this reaction would be irreversible.

BORG and COTZIAS (1962c) further stated that, when the free radical was not stabilised by concentrated acid, spontaneous "dismutation" occurred into an equimolar mixture of the original phenothiazine derivative and the phenazothionium ion (I) which would presumably give rise at once to the sulphoxide II, according to the equation in Fig. (1)

The possible formation of a free radical ion by the action of certain oxidants on phenothiazine derivatives has also been considered by FORREST, FORREST & BERGER (1958) in a note on the metabolites of drugs derived from phenothiazine. In a later communication, PIETTE & FORREST (1962) studied the electron paramagnetic resonance of free radicals in the oxidation of drugs derived from phenothiazine *in vitro* and concluded that the first product obtained by oxidation of phenothiazine derivatives is a positive ion radical and that this radical is responsible for the coloured reaction products observed.

It was observed by FELS & KAUFMAN (1959), that addition of concentrated sulphuric acid to (presumably an aqueous solution of) the sulphoxide of chlorpromazine yielded a "red reaction product". Working with fluopromazine, STREET (1962a) also suggested that the sulphoxide of fluopromazine reacts with 50 % sulphuric acid to yield a solution whose ultraviolet absorption curve is identical with that given by fluopromazine itself in 50 % sulphuric acid. Further support for the supposition that the sulphoxide might be converted to a coloured radical is also contained in the article by BORG & COTZIAS (1962c), who suggested that the equation in Fig. (1) (*vide supra*) might be reversed at a sufficiently high hydrogen ion concentration. They noted that such a reaction could explain the formation of identical chromophores from chlorpromazine and from its sulphoxide in the presence of concentrated acid, which had been reported by previous workers.

While investigating the comparative colorimetry and ultraviolet spectrophotometry of chromophores of these types, prepared from various phenothiazine derivatives, we noticed a paper by LUCAS & FABIERKIEWICZ (1963) concerning some tests for identifying phenothiazine tranquillizers. They stated that their most important contribution appeared to be in a colour reaction involving bromine and concentrated sulphuric acid, which with phenothiazine derivatives (except trifluoderivatives) produced an immediate stable violet colour having an absorption maximum at 580 mμ. Trifluo-compounds gave a red colour with a peak at 540 mμ. They gave no details of the ultraviolet absorption spectra of these solutions.

We were greatly interested in this reaction because it seemed to us that at least one function of the bromine would be that of electron-acceptor. This removal of a single electron from the parent phenothiazine derivative would yield a free radical ion, which itself could give rise to a sulphoxide, by loss of one electron. But the bromine might have a further role to play. It might also brominate the phenothiazine derivative. Such bromination could perhaps take place in the parent compound, in much the same way as phenols are brominated, or it could take place subsequently. Further the presence of concentrated sulphuric acid might give rise to a free-radical ion of this brominated phenothiazine derivative.

In an attempt to explain these observations, and because we felt that some further light might be cast on the problem of identifying metabolic phenolic derivatives of phenothiazine, we have studied the products of bromination of a number of phenothiazine tranquillizers.

Experimental Procedures

The procedures described below were carried out. When details of a procedure have already been published, only the reference to the relevant literature is given.

De-ionised water was used throughout.

I. *Treatment of phenothiazine derivatives with bromine*

For their "bromination-acidulation spot plate test on solid material" LUCAS & FABIERKIEWICZ (1963) recommend the use of a saturated solution of bromine water prepared by adding 25 ml. of liquid bromine to 100 ml. of water containing 10 ml. of chloroform. This mixture is allowed to stand for 3 to 4 days during which it is shaken at least 10 times daily.

We have used the simpler but equally effective procedure involving liberation of bromine by acidification of a bromate bromide mixture, which was prepared by dissolving 3 grams of potassium bromide and 0.835 grams of potassium bromate in 100 ml. of water. This solution was labelled "Brominating Mixture A". "Brominating Mixture B" was prepared by making a (1 + 9) volume dilution of "A" with water.

Procedure

Dissolve 60 mg. of phenothiazine derivative in 60 ml. of 5 N-sulphuric acid, and divide the solution into 6 × 10 ml. portions, in tubes labelled a, b, c, d, e and f. Add "Brominating Mixture B" and 5 N-sulphuric acid to these tubes as given below –

Tube	Volume (ml) of Brominating Mixture B	Volume (ml) of 5N-H_2SO
	1.13	8.87
b	2.26	7.74
c	3.39	6.61
d	4.52	5.48
	5.56	4.44
f	6.78	3.22

Mix the contents of each tube, and allow it to stand for 30 minutes. To each solution add 15 ml of 60% potassium hydroxide, cool and shake with 30 ml of ether in a separating funnel. After the layers have separated, run off and discard the lower aqueous phase, and dry the ether by shaking with about one g. of anhydrous sodium sulphate. Transfer 0.3 ml of ether extract to a tube. Then carefully evaporate this and the remaining ether extract to dryness on a boiling water bath.

II. *Preparation of bromophenothiazine derivatives.*

Dissolve 50 mg of phenothiazine derivative in 50 ml of 5 N-sulphuric acid. Add 3.5 ml of "Brominating Mixture A" (supra). Mix, and allow to stand for 30 minutes. Add 25 ml of 60% potassium hydroxide, cool, and shake in a separating funnel with 200 ml of ether. After the layers have separated, discard the aqueous layer and wash the ether phase with 10 ml of 0.1 N-sodium hydroxide. Shake the washed ether extract with 50 ml of 0.1 N-sulphuric acid. Remove the aqueous phase. To this aqueous phase add 11 ml of N-sodium hydroxide, and shake with 100 ml of ether. Separate and dry the ether solution with sodium sulphate. Evaporate to dryness 2 × 1 ml portions of this dried ether extract separately on a boiling water bath. Use the residues obtained for plotting the absorption curve in 0.1 N-sulphuric acid and for chromatography.

Evaporate the remainder of the dried ether extract to dryness on a boiling water bath.

III. *Chromatography of products of bromine treatment*

Dissolve each of the residues, obtained as described under Section I above, in 10 drops of chloroform, and carry out reversed phase paper chromatography as described by STREET (1962b), in an oven maintained at 95°C. Use a mobile phase of 0.2 M acetate/hydrochloric acid buffer pH 4.6, and a running time of 17 minutes. After chromatography dry the papers in a stream of warm air. Locate the spots on the papers first by inspection in 254 mμ radiation and then by immersion in cold 50 per cent sulphuric acid.

IV. *Elemental Analyses*

The total halogen content of the phenothiazine derivatives was determined quantitatively by the oxygen-flask method of SCHÖNIGER (1955 & 1956), as described by MACDONALD (1961) and by HASLAM, HAMILTON & SQUIRRELL (1961). A specific qualitative test for the presence of bromine was also carried out, as described by the latter workers, on the absorbing solution in the flask after combustion of the sample in oxygen.

Samples weighing 10 to 20 milligrams were used with a platinum gauze (1.5 cm × 3 cm.) in a 500 ml. Quickfit round-bottomed flask containing 5 ml of N-sodium hydro-

xide. After combustion, the stoppered flask was set aside for 15 minutes, and the contents were transferred quantitatively with water into a 25 ml measuring cylinder After thorough mixing, 1 ml. portions of this solution were diluted to 5 ml with water and used for analysis. The absorption spectrum of the final coloured solution obtained by carrying out the procedure for total halogen content was plotted from 400 mμ to 550 mμ against a blank taken through the entire oxygen flask procedure. Calculations of halogen content were made from readings taken at 470 mμ.

Results

The pattern obtained after chromatography of mixtures a to 'f' (see Section I of Experimental Procedures) is illustrated in figures 2 and 3 for promazine and chlorpromazine respectively With chlorpromazine, the first stage in the action of bromine yields two spots, – one with an R_f value corresponding to that of the unchanged compound and one with an R_f value corresponding to that of the sulphoxide Both spots appeared as a pink colour on treatment with 50 % sulphuric acid. Further action of bromine as shown by runs b c d and e on the chromatogram, gave rise to two further spots besides those seen in a One of these additional spots had an R_f value of 0 7 and gave a violet pink colour with 50 % sulphuric acid the other R_f value 0 55 showed up as a mauve colour in 50 % sulphuric acid Finally in f there was almost complete conversion to the compound of R_f 0 55 with only traces of two other spots, one of R_f 0 7 and one at the origin.

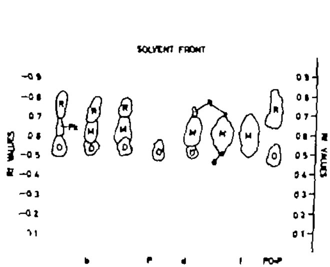


Fig. 2. Tracings of a chromatogram of products obtained by treating Promazine with bromine (7 minutes run in solvent of acetate/HCl buffer pH 4 6 at 95 C. Whatman No 3 paper impregnated with Tributyrin.
P = Promazine PO = Promazine sulphoxide. For significance of a b c' etc., see text. Compounds located on paper first 254 mμ light and then by dipping in 50 per cent H_2SO_4. Colours in 50 % H_2SO_4: – Pk = Pink O = Orange R = Red M = Mauve Vl/Pk = Violet/Pink.

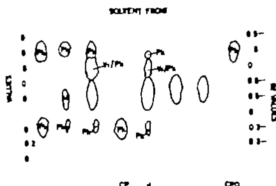


Fig. 3. As for Figure 2, but for Promazine read Chlorpromazine.
CP = Chlorpromazine CPO = Chlorpromazine sulphoxide. Colours in 50% H_2SO_4 :– Pk = Pink, O = Orange R = Red M = Mauve; Vi/Pk = Violet/Pink.

Promazine yields results similar to those of chlorpromazine (see fig. 2), the final product under 'I' showing essentially a single spot of R_f 0.62, which appeared as a mauve colour on treatment with 50 / sulphuric acid.

Similar results for chromatography of the 'I' solutions were obtained with mepazine, prochlorperazine and laevomepromazine.

The large-scale preparation of the bromochlorpromazine derivative yields a product showing on chromatography a single spot of R_f value 0.55, corresponding to that found in the 'I' mixtures. Figures 4 and 5 show

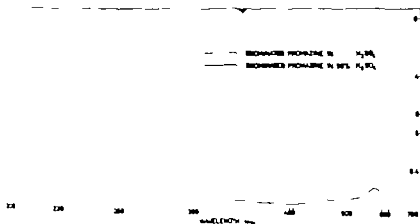


Fig. 4. Ultra-Violet absorption spectra of the 'I' mixture (see text) of Promazine in 0.1N–H_2SO_4 (broken line) and in 50 per cent H_2SO_4 (continuous line).

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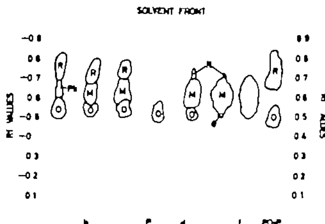


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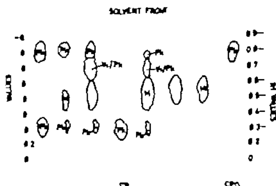


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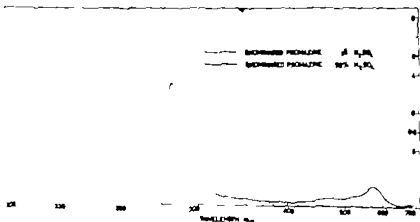


Fig. 4. Ultra-Violet absorption spectra of the 'I' mixture (see text) of Promazine in 0.1N–H_2SO_4 (broken line) and in 50 per cent H_2SO_4 (continuous line).

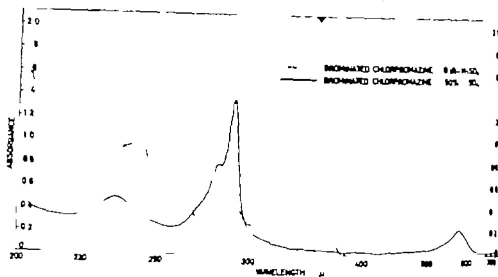


Fig. 5 As for Figure 4 but for Promazine read Chlorpromazine.

the ultraviolet absorption spectra of such products obtained from Promazine and Chlorpromazine, respectively In 0 1 N sulphuric acid, the curve is of a shape similar to those of the spectra of sulphoxides of phenothiazine derivatives (see Fig. 6 curve (b)) with an additional peak around 256 mμ. In this medium the solution is colourless. The solution in 50 / sulphuric acid is blue in colour besides displaying a maximum at 580 mμ, it shows a sharp peak at 287 mμ with a shoulder at 280 mμ. This peak is similar in shape, but not in wavelength, to that obtained by plotting the absorption of the red-coloured solution prepared by mixing chlorpromazine with 50 / sulphuric acid which curve is shown for comparison purposes in Figure 6 (a)

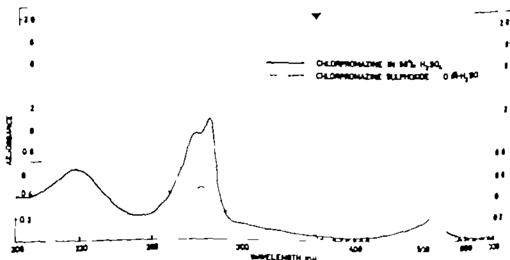


Fig. 6. Ultra-violet absorption curve of (a). Chlorpromazine in 50 per cent sulphuric acid (continous line) and (b) chlorpromazine sulphoxide in 0.1N-sulphuric acid (broken line).

Table 1

Compound	Molecular Weight	Qualitative Test for presence of Br after combustion	Weight (mg) used for O_2 Flask (A)	(A) as fraction of mMW	Measured absorbance*)	Absorbance adjusted for 1/20 mMW (B)	(B) $\times \frac{2}{0.82}$ i.e. with chlorpromazine hydrochloride as standard	Number of Halogen Atoms per Mole Deduced to be Present: Total Halogen Atoms	Number of Halogen Atoms per Mole Deduced to be Present: Bromine Atoms
Promazine Hydrochloride	321	Negativ	16	1/20	0.48	0.48	1.1	1	0
Dibromopromazine Sulphoxide (free base)	460	Positive	11	1/20 × 0.48	0.43	0.89	2.1	2	2
Chlorpromazine Hydrochloride	355	Negative	17.8	1/20	0.82	0.82	2		0
Dibromochlorpromazine Sulphoxide (free base)	494	Positive	13	1/20 × 0.51	0.70	1.31	3.1	3	2

*) All measurements were made at 420 mμ against a "reagent blank"

Results of the elemental analyses for halogens are summarised in table 1

The infra-red spectrum of the brom-chlorpromazine derivative was plotted as a thin film deposited on a sodium chloride plate and is shown in fig. 7

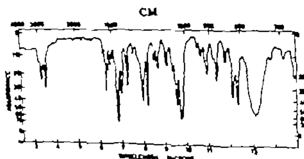


Fig. 7 Infra-red absorption spectrum of product obtained by treating chlorpromazine with excess bromine.

Discussion

The results of chromatographing the product obtained by treating the phenothiazine derivatives with bromine suggest that the first step in this reaction is the formation of a sulphoxide. With chlorpromazine the second step is the formation of two extra compounds from the sulphoxide. As increasing amounts of bromine are added, the compound with the higher R_f value disappears leaving (apart from a very small spot of R_f 0 7) a single spot on the chromatogram at R_f 0 55 This compound (R_f 0.55) has been prepared in larger quantities and some of its properties have been examined. Ultraviolet absorption measurements of the compound in 0 1 N-acid (see fig. 5) yield a curve resembling that given by the sulphoxide of chlorpromazine (see fig. 6 curve (b)) but with significant shifts of peak wavelengths towards higher wavelengths. In 50 % sulphuric acid, the ultraviolet absorption curve (see fig. 5) resembles that given by the solution obtained by treating the sulphoxide of chlorpromazine with 50% sulphuric acid (*i e* that of the free radical of chlorpromazine), (see fig. 6 curve (a)) but again with significant shifts of the peaks towards higher wavelengths

The results of elemental analyses for halogens (by the oxygen flask method) indicate that the compound of R_f 0 55 contains two bromine atoms per molecule It is therefore suggested that this compound (R_f 0.55) may well be the sulphoxide of a dibromo-derivative of chlorpromazine. Further it is tentatively suggested that the compound of R_f value 0.7 may be the sulphoxide of a monobromo-derivative of chlorpromazine. In the type of chromatography used, R_f values of 0 8 0 7 and 0 55 could be consistent with the sulphoxide of a phenothiazine derivative containing 0 1 and 2 extra halogen atoms per molecule, respectively *i e* the R_f value would decrease as more bromine atoms were incorporated into the molecule.

Additional evidence suggestive of the presence of a sulphoxide group is provided by plotting the infra red spectrum. SALZMAN & BRODIE (1956) considered that a strong band appearing at 1020 cm^{-1} in the spectrum of their metabolite of chlorpromazine, but not in that of chlorpromazine itself was possibly due to the presence of a sulphoxide group on the ring. The infrared spectrum of our brominated compound (see fig. 7) shows a strong band at 1020 cm^{-1}

The ease with which bromination occurs in these phenothiazine derivatives may have some bearing on the formation of the phenolic urinary metabolites of chlorpromazine studied by FISHMAN & GOLDENBERG (1963)

Summary

This article describes the effect of addition of bromine to various phenothiazine derivatives. The products resulting from this addition have been investigated by chromatography ultraviolet and infrared spectrophotometry and reaction with 50% sulphuric acid. The results suggest that chlorpromazine reacts with bromine to give, initially chlorpromazine sulphoxide, and that the sulphoxide is then brominated first to a mono- and then to a di-bromochlorpromazine sulphoxide.

The ease with which bromination occurs may be of assistance in unravelling the problems connected with the formation of phenolic metabolites of phenothiazine derivatives.

Acknowledgement

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Inhibition by 5-Hydroxytryptophan of Insulin-Induced Adrenaline Depletion

By

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(Received April 13, 1964)

By a fluorescence method for histochemical detection of certain catecholamines and tryptamines, descending monoaminergic nerve tracts have been demonstrated in the spinal cord, (CARLSSON, FALCK, FUXE & HILLARP 1964). The cell bodies are localized in the brain stem, and the terminals are highly concentrated *i. a.* in the sympathetic lateral column. In the mouse the pathways containing 5-hydroxytryptamine (5-HT) seem to dominate quantitatively over those containing noradrenaline (NA). These findings prompted us to investigate the influence of 5-hydroxy tryptophan (5-HTP) a precursor of 5-HT on the depletion of adrenaline (A) caused in the mouse adrenals by insulin hypoglycemia.

Material and Methods

White mice of either sex weighing between 20 and 35 g were used. All animals except those in the normal group were fasted from about 18 hours before the experiment, but had free access to water Insulin was given intraperitoneally in a dose of 50 IU/kg 7 hours before killing the animals. The 5-HTP was injected subcutaneously in dose of 200 mg/kg (DL-form) 30 minutes before the insulin and then 100 mg/kg every 90 minutes. In order to investigate the possibility that the effect of 5-HTP was due to peripheral action, we also ga o 5-HT which does not penetrate into the central nervous system. It was administered subcutaneously at a dose of 50 mg/kg (free base) 30 minutes before the insulin and then 25 mg/kg every 3 hours. The mice were beheaded. The adrenals were dissected out as quickly as possible and homogenized in 0.4 N perchloric acid. Differential determination of A and NA was performed spectrophotofluorometrically (BERTLER, CARLSSON & ROSENGREN 1958). In some experiments 0.4–0.5 ml samples of blood from the cut carotid arteries were collected for determination of 5-HT and glucose. The amounts of 5-HT in the adrenals and blood plasma (EDTA as anticoagulant) were determined spectrophotofluorometrically after cation exchange chromatography (BERTLER 1961). Blood glucose concentrations were measured colorimetrically after condensation with o-toluidine (HULTMAN 1959).

Table 1

The adrenal contents of adrenaline (A) noradrenaline (NA) and 5-hydroxytryptamine (5-HT) and the blood concentrations of 5-HT and glucose in unfasted and fasted mice as well as in mice treated with insulin 5 hydroxytryptophan (5-HTP) plus insulin or 5-HT plus insulin. All values given are means ± s. e. m. The figures in brackets indicate number of experiments.

	Adrenals			Blood	
	A in μg/pair	NA in μg/pair	5-HT in μg/pair	5-HT in μg/ml blood	Glucose in mg/100 ml blood
Unfasted	4.6 ± 0.52 (9)	3.2 ± 0.52 (9)	0.0 (3)	—[1]	130 ± 3.3 (8)
Fasted	5.7 ± 0.37 (12)	4.0 ± 0.28[4] (12)	0.0 (3)	—[1]	68 ± 6.0 (7)
Insulin	2.4 ± 0.15 (21)	3.1 ± 0.19 (21)	0.0 (3)	0.0[2]	37 ± 3.2 (14)
5-HTP + Insulin	4.3 ± 0.34[3] (19)	2.5 ± 0.21[4] (19)	1.9 ± 0.31 (8)	0.95[2] 1.7[2]	36 ± 3.2 (14)
5-HT + Insulin	2.8 ± 0.40[5] (13)	2.5 ± 0.36[5] (13)	1.6 ± 0.34 (8)	4.2[2] 3.8[2]	20 ± 6.2 (14)

[1]) Analysis not performed.
[2]) Single value.
[3]) Highly significantly ($p < 0.001$) different from the insulin group.
[4]) Almost significantly ($0.02 < p < 0.05$) different from the insulin group.
[5]) Not significantly ($p > 0.05$) different from the insulin group.

Results and Discussion

In agreement with earlier findings, insulin induced hypoglycaemia produced a marked depletion in adrenal A and a moderate one in adrenal NA (HÖKFELT 1951 OUTSCHOORN 1952) (table 1) Treatment with 5-HTP significantly reduced the depletion of A. This effect was probably not due to a peripheral site of action since injections of 5-HT were inefficient by this criterion in spite of the fact that the blood concentrations of 5-HT after 5-HT treatment were higher than after 5-HTP treatment In the doses employed 5-HT but not 5-HTP enhanced insulin hypoglycemia (KOBAYASHI UI & WARASHINA 1960) Evidently 5-HTP did not prevent A depletion in the adrenals by counteracting insulin hypoglycemia. In fact the hypoglycemic symptoms tended to be emphasised by 5-HTP with an increased number of deaths.

After treatment with 5-HTP and 5-HT there was an accumulation of 5-HT in the adrenals. The equal or larger uptake after 5-HTP despite a lower blood concentration indicates that the amino acid enters the adreno-medullary cells more easily than the amine The 5-HT has been shown to

be taken up in the adrenomedullary granules *in vivo* (BERTLER, ROSENGREN & ROSENGREN 1960). Maybe the decrease in NA content after 5-HTP and 5-HT is at least partly due to displacement by 5-HT

The 5-hydroxytryptaminergic fibres to the sympathetic column of the spinal cord descend mainly in the dorsal half of the lateral funiculus (CARLSSON, FALCK, FUXE & HILLARP 1964), where the inhibitory pathway to the sympatho-adrenal preganglionic neurons is localized (LIM, WANG & YI 1938, ALEXANDER 1946 UVNÄS 1960) The excitatory nerve tract is localized in the ventral half The reduced A secretion after 5-HTP may be explained if 5-HT acts as an inhibitory transmitter to the sympatho-adrenal preganglionic neurons. The A hypersecretion after insulin is regulated from a region in the brain stem caudal to the superior colliculi (CRONE 1963). There may be 5-hydroxytryptaminergic nerve terminals in this area (FUXE, unpublished results, 1964) The findings presented here do not therefore exclude the possibility that the reduced A secretion after 5-HTP is due to a supraspinal mechanism. However the spinal 5-hydroxytryptaminergic mechanism is much more likely to be responsible for the observed phenomenon, as it is situated distally to the supraspinal mechanism.

Summary

Treatment of mice with 5-hydroxytryptophan markedly inhibited the insulin-induced depletion of adrenaline in the adrenals. Since 5-hydroxytryptamine does not produce this effect, it is probably due to a central nervous mechanism. It is likely that the 5-hydroxytryptamine in the lateral horns of the spinal cord serves as an inhibitory transmittor to the sympatho-adrenal preganglionic neurons.

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The Uptake of Glucose by Acute Inflammatory Oedema

By

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(Received April 16, 1964)

Inflammation induces characteristic changes in the permeability of the capillary wall to macromolecules. The phenomenon can be investigated by comparing the uptake by the skin from the blood of substances of different molecular weights at various times after initiation of the inflammatory reaction. In previous studies the exchange of water electrolytes and serum albumin between oedema fluid and plasma was studied at different stages of the inflammatory process.

During the initial phase of inflammation the demand for energy increases in the inflamed tissue. A specific uptake from the blood of potential energy-producing substances might therefore be expected. The problem has been attacked by studying the uptake of ^{14}C-glucose by inflamed skin.

Methods

Male albino mice of a single strain, weighing 22–26 g, were used. The fur on the back was cut with an electric clipper and the skin depilated with barium sulphide. On the next day 50 µl of a ^{14}C-glucose solution containing approximately 1 µC (uniformly labelled, The Radiochemical Centre, Amersham, England) was injected into a tail vein. Five minutes later two symmetrical semilunar areas were marked out on the skin under fluothane ® (halothane B.P.) anaesthesia (cf. Szporny Langgård & Hvidberg 1964). Within one of the areas acute inflammation was provoked by applying filter paper moistened with xylene. One minute, 5 minutes or 2 hours after ending the xylene treatment the animal was stunned by blow on the neck. The left carotid artery was cut, and 250 µl of blood were sampled by means of a Carlsberg construction pipette prepared with heparin saline. After decapitation and bleeding, the two areas with their underlying subcutaneous tissue were excised and weighed on a torsion balance. 1 olume 3 times the weight of the tissue N/2 sodium hydroxide was added immediately and the samples wer heated to 100°C for 10 minutes to obtain tissue destruction. The proteins were precipitated by adding the same olumes of a 10 / zinc sulphate solution. Plasma samples were treated in an identical way After centrifuga-

tion 100 µl of the clear supernatant were added to 4 ml of the scintillation medium described by BRAY (1960) The radioactivity was measured in a liquid scintillation counter (Isotope Developments LTD)

Knowing the dilution factor and the weights of the total samples, the total radioactivity of the skin samples could be calculated. The weight and radioactivity of the oedema were determined as the difference between the oedematous skin sample and the corresponding control sample (cf LANGGÅRD SCHOU SZPORNY & HVIDBERG 1964) The ratio between radioactivity per unit weight oedema and per unit weight plasma was calculated. This ratio should be exactly one if the oedema is due simply to an extravascular accumulation of plasma.

The percentage recoveries of radioactivity from skin and plasma samples were determined by adding known amounts of ^{14}C-glucose to skin and plasma samples. No statistically significant difference was found between the efficiency of measuring ^{14}C glucose added to 14 skin samples and 10 plasma samples ($p > 0.1$ by t-test).

Results

In table 1 the animals killed 5 minutes after induction of oedema are listed according to the weight of the oedema, in descending order The total radioactivity of the oedema and the activity per mg of the corresponding plasma sample are indicated in the table. The ratio between counts per minute per mg oedema and counts per minute per mg plasma was calculated for each individual animal. The average ratio was 0.97 indicating that a dynamic equilibrium existed between oedema glucose and plasma glucose during the initial phase of oedema formation.

Table 1

The ratio between radioactivity of experimentally induced acute inflammatory oedema and plasma 5 minutes after intravenous injection of ^{14}C glucose.

Weight of animal (g)	Weight of oedema fluid (mg)	Counts per sec. of oedema fluid	Counts per sec. of 1 mg plasma	Cps per mg oedema fluid / Cps per mg plasma
23 5	92	104	1 10	1 03
24 5	92	84	0.85	1 07
24 0	92	92	0.89	1 12
25 5	56	60	1 12	0 96
22.0	56	37	0.75	0.88
22.5	55	33	0.63	0 95
26.0	48	22	0 68	0.68
24 0	42	56	1 18	1 13
26 0	36	31	0 71	1 21
22.0	31	19	0 94	0 66
			Average	0.97
			s. e. m.	± 0 059

Table 2

Difference between total radioactivity of the treated sample and the corresponding control sample 1 minute after initiation of inflammatory process. *In these animals no oedema* had yet developed, as indicated by the insignificant weight difference between the two samples. For comparison similar values obtained from 7 animals injected with ^{131}I-labelled human serum albumin instead of ^{14}C-glucose are included (unpublished results from previous study).

	cps (treated side) − cps (control side)			cps per mg plasma
	mean		s.e.m.	mean
^{14}C glucose (= 7)	−1	±	4.5	1.0
^{131}I-albumin (n = 7)	14	±	9.5	3.6

In 7 animals killed 1 minute after initiation of the inflammatory process, no oedema had yet developed, as indicated by the *insignificant difference* in weight between the two samples. In these animals no difference between the total radioactivity of the two samples was found, in contrast with the findings on animals injected with ^{131}I-albumin instead of ^{14}C-glucose (table 2). (The ^{131}I-albumin findings, unpublished, were obtained in an earlier study (LANGGÅRD, SCHOU SZPORNY & HVIDBERG 1964).

In the experimental circumstances used, the size of the oedema reached its maximum 2 hours after initiation of oedema. At this time practically all of the injected glucose has been removed from the plasma and from the oedema by way of total distribution and metabolism. Low counting rates were therefore obtained on samples from 10 animals killed at this hour *but no signs of accumulation of glucose during the late phase were* recorded.

Discussion

Previous studies have indicated that acute inflammatory oedema fluid is a bulk filtrate of plasma (LANGGÅRD, HVIDBERG & SZPORNY 1964) except during the initial 15 minutes of the inflammatory process when the albumin content is lower than in plasma (ASCHHEIM & ZWEIFACH 1962 LANGGÅRD, SCHOU, SZPORNY & HVIDBERG 1964) From then on, however the protein content also can be accounted for in terms of extravasated plasma. Most likely an ultra-filtrate of plasma is formed initially Shortly after however the capillary wall becomes permeable to all plasma components, and 15 minutes after inducement of oedema a dynamic equilibrium exists between oedema and plasma proteins. This concept is substantiated

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22.0	31	19	0 94	0 66
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by the study reported here, with the finding of a complete equilibrium between oedema and plasma glucose 5 minutes after initiation of the inflammatory process.

The uptake of glucose from the blood by inflamed tissue is, however of interest also from another point of view It is well known that some substances do not always move according to concentration gradients or gradients of electrochemical potentials The significance of energy supply for active transport has been emphasized by ROSENBERG (1954). YOFFEY & COURTICE (1956) showed that protein leakage through the capillary wall is greatest in tissues with a high protein metabolism. ARAKI (1957) pointed out that the initial local reaction to trauma could be an activation of intracellular regulators, such as the mitochondria, in order to increase energy utilization by the stimulated tissue cells. On the basis of these theories an excessive uptake of glucose, as the most important source of energy might be expected in inflamed tissue. By the method applied however no accumulation of glucose could be demonstrated, either in the initial phase or later In a previous study in which ^{131}I-albumin was circulating before treatment with xylene, an accumulation of radioactivity preceding oedema formation could be demonstrated at the site of inflammation (LANGGÅRD SCHOU SZPORNY & HVIDBERG 1964) This phenomenon was thought to be due to the so-called "endothel activation (JANCSÓ 1961) The figures in table 2 indicate that the phenomenon is specific for protein and that glucose accumulation does not constitute part of the initial inflammatory response.

Summary

The uptake of glucose from the blood by inflamed skin has been studied in mice after intravenous injection of ^{14}C glucose. An accumulation of glucose at the site of inflammation did not precede oedema formation and could also not be demonstrated at later stages of the inflammatory process. Five minutes after initiation of the inflammatory process a state of dynamic equilibrium existed between glucose in oedema fluid and plasma.

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Acetylcholine in the Brain of Morphine Tolerant and Non-Tolerant Rats

By

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(Received April 27 1964)

Many authors from SLAUGHTER and his associates (1940 a, b, c) to GORDONOFF (1963) have argued in favour of a cholinergic mechanism of morphine and related substances. Recent investigations have, however cast serious doubt on this hypothesis about its analgesic action (JÓHANNESSON 1962 JÓHANNESSON & SCHOU 1963) On the other hand, the results of SCHAUMANN (1956 1957) and PATON (1957) showing that morphine can reduce or inhibit the release of acetylcholine from nerve endings in the isolated guinea pig intestine, indicate that morphine may somehow interfere with cholinergic mechanisms and probably affect the acetylcholine content of the tissue These authors did not measure acetylcholine contents in the intestine, nor did they perform experiments on intestines from morphine tolerant animals.

In the literature available we have found no records of experiments in which the brain levels of acetylcholine were compared in morphine tolerant and non tolerant animals. In our investigation we have therefore determined the amounts of acetylcholine extracted from brains of morphine tolerant rats at two intervals after the last dose of morphine. We endeavoured thereby to measure acetylcholine in the brains of tolerant rats in the excitatory state as well as in the early abstinence period (cf MARTIN *et al* 1963) For comparison we determined the levels of acetylcholine in the brains of rats that had never received any drugs and also in the brains of rats that had been given one injection of morphine.

Material and Methods

Animals For these experiments we used male Holtzman rats (190–280 g), kept under constant environmental conditions and having free access to water and a commercial food preparation Normal rats are below described as non-tolerant.

Morphine tolerance in the rats was produced by subcutaneous injections of morphine in the morning and in the evening of each day in increasing amounts for approximately

4 weeks. Each injection amounted to half the daily dose of the drug. The initial dose was approximately 12.5 mg/kg a day and the final daily dose was about 75 mg/kg. These doses refer to morphine as the base. The rats usually gained 10–20 g in weight during this period. The tolerant rats were killed either 2 or 20 hours after the last injection of morphine.

Injections were given in the gluteal region, preferably in the midline. The injection solution was 38 mg/ml of morphine sulphate in water.

Experiments. Seven rats that had never received drugs of any kind were anaesthetized with ether and 5–6 ml of blood were withdrawn from the abdominal aorta. Then, while the animals were still alive, the neck was cut open just below the occiput of the skull, the skull opened in the sagittal line and the total brain (including the brain stem and the cerebellum) immediately removed with a forceps for acetylcholine determination (see below).

The same procedure was applied to the tolerant rats. Fourteen tolerant rats were injected with approximately 37 mg/kg of morphine on the evening of the last day in the tolerance period. Seven of them were killed for analysis 20 hours later whereas the remaining seven rats were then reinjected with this dose of morphine and killed 2 hours later.

Finally seven non-tolerant rats were given 37 mg/kg of morphine. Only three of them were alive 2 hours later. These were then killed and their brains were removed for acetylcholine determinations as described.

Bioassay of acetylcholine was performed, with slight modification, as described by MAC INTOSH & PERRY (1950). The brain tissue was frozen in liquid nitrogen immediately after being removed, ground with mortar and pestle and extracted with trichloroacetic acid, and the acetylcholine contents were measured by an eviscerated cat blood-pressure method. Three doses of acetylcholine bromide (increasing at 0.3 log. intervals) were used for the reference curve and three different volumes (at 0.3 log. intervals) of the extract were injected into the animal. The amounts of acetylcholine in the samples were then determined from the reference curve. The estimated acetylcholine contents of each brain was obtained by averaging the amounts found in the three samples and expressed as µg acetylcholine per g wet brain.

The depressor responses were blocked by atropine sulphate.

The results were analysed statistically by Student's "t" test (SNEDECOR 1956).

Results

The results are given in table 1. The concentrations of acetylcholine was on an average between 2.0 and 2.2 µg per g wet brain in 3 out of 4 groups of rats. In the fourth group the morphine tolerant rats killed 20 hours after the last dose of morphine, acetylcholine appeared to be present in lower amounts in the brain, with a mean concentration being 1.88 µg/g. None of the mean values differed, however, significantly from one another ($P > 0.05$).

Discussion

In our investigation no attempt was made to differentiate between the various parts of the rat brain or to measure their contents of acetylcholine separately. The results therefore refer to the acetylcholine contents of whole brains.

Table 1

Acetylcholine contents of wet brain (µg/g ± standard deviation) of morphine tolerant and non-tolerant rats, with P values for differences from controls.

Experiments	No Animals	Acetylcholine (µg/g ± s.d.)	P Values
Controls	7	2.18 ± 0.38	
Morphine (2 hr)[1]	7	2 04 ± 0.23	0.3–0.5
Morphine (20 hr)[1]	7	1 88 ± 0.25	0.1–0.2
Morphine (2 hr)[2]	3	2.12 ± 0.12	0.1–0.8

[1] Morphine tolerant rats given the last dose of morphine 2 or 20 hours before acetylcholine extraction.

[2] Non-tolerant rats that received morphine (37 mg/kg) 2 hours before acetylcholine extraction

In the brains of rats killed without any previous administration of drugs, the brain concentration of acetylcholine was on an average 2.2 µg/g (table 1) This is in a good agreement with the results of HERKEN *et al.* (1957) They used albino rats of both sexes and found in their control group a mean concentration of 2.4 µg acetylcholine per g total brain. Their test object was the rectus muscle of the frog.

HERKEN *et al* administered 10 mg/kg and 200 mg/kg of morphine by intravenous injections to their rats. The animals were killed 15–20 minutes later The acetylcholine contents of the brain were significantly increased after the higher dosage of morphine, but not after the lower dosage. The increased amounts of acetylcholine in the brain after the higher dose could be due to a resultant cholinesterase inhibition In our experiments the non tolerant rats were given a dose of morphine far lower than the higher dose of the drug given by HERKEN *et al* It was therefore to be expected that we should not find increased amounts of acetylcholine in the brains of the three non tolerant rats given morphine (table 1)

When morphine is administered to morphine tolerant rats, they will be found in an excitatory state 2 hours after the injection, whereas exhaustion is the predominant feature 20 hours later This was found by MARTIN *et al* (1963) and was also apparent in our rats.

The levels of brain acetylcholine were found to be approximately the same, whether the tolerant rats were killed 2 or 20 hours after the last injection of morphine in fact they did not differ significantly from those found in the brains of rats that had never received morphine. The rats were highly tolerant to the analgesic effect of morphine at both times after the last injection in the tolerance period (our own unpublished results) as well as to the lethal effect of the drug (cf experiments). It there-

fore seems unlikely that acetylcholine in the brain is causally connected with the analgesic action of the drug or with the signs and symptoms of morphine tolerance and abstinence in the rat. It should, however be recalled that all the available methods for determining the amounts of acetylcholine in tissues, are very crude. Morphine might thus affect significantly the amounts of acetylcholine at certain sites of action in the tissues, even though it could not be detected by a general extraction of the denaturated tissue samples.

The reduced release of acetylcholine from the small intestine of the guinea pig caused by morphine, as shown by SCHAUMANN (1956, 1957) and PATON (1957), could be a phenomenon peculiar to the intestines. In this connection the experiments of LA BARRE (1924) should be mentioned. He found, in experiments on the intestines of the rabbit, that the effect on the gut of morphine (among other opium alkaloids) was connected with the amounts of choline in the tissue. These experiments could therefore indicate that morphine somehow interacts with cholinergic substances in the intestines.

Summary

Acetylcholine was determined in the total brains of morphine tolerant rats, of non-tolerant rats given one injection of morphine and of rats that had never received morphine. The gross brain concentrations of acetylcholine were not significantly different in these groups.

The results indicate that acetylcholine in the rat brain is neither causally connected with the analgesic action of morphine nor with the development of morphine tolerance and abstinence symptoms in this species.

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Chronic Toxicity Studies on Phenacetin, N-Acetyl-p-Aminophenol (NAPA) and Acetylsalicylic Acid on Cats

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The compatibility of phenacetin during chronic administration has been the subject of active discussion. Severe affections of man after continuous ingestion of preparations containing phenacetin have been reported. Phenacetin is frequently replaced by N-acetyl-p-aminophenol (NAPA). This substance, apart from p-phenetidin, which has a strong toxic effect, is an intermediary metabolite of phenacetin. It is assumed to be the actual effective pharmacological principle of phenacetin, but of lower toxicity.

This paper reports results obtained in animal experiments and is a contribution to the discussion on the relative toxicity of phenacetin and NAPA on chronic administration. Since acetylsalicylic acid, which was introduced long ago as an analgesic antipyretic, appears to be gaining in importance compared with preparations containing phenacetin, it was included in the studies.

Methods

Cats were used as test animals, since they are especially sensitive to changes in blood pigments (Lester 1943).

A preliminary study showed that a daily dose of 200 mg/kg phenacetin, NAPA or acetylsalicylic acid administered orally on three successive days was too high. At the end of this study the cats that had received acetylsalicylic acid showed the most rapid recovery.

In the chronic toxicity study on 18 cats, the three test substances were administered in smaller doses:

Group A	(Cat A 1 to A 6) acetylsalicylic acid	for 4 weeks 25 mg/kg daily
Group P	(Cat P 1 to P 6) phenacetin	subsequently for 22 weeks 50 mg/kg daily
Group N	(Cat N 1 to N 6) NAPA	

Owing to their insolubility in water to increase the possibility more effective administration the substances were administered in the form of tablets or pieces of tablets. Each tablet contained 200 mg test substance.

At the beginning of the experiment the animals, after several weeks observation, were found to be healthy and considered suitable for experimental use. During the whole investigation the animals were held in roomy boxes, separated into groups. Male and female animals were evenly distributed in the groups and identified by earmarks. During the entire experimental period they received a standard diet, consisting, apart from milk, of raw horse meat or horse-meat stewed with rice. The body weight of the cats was checked weekly

Blood and urine examinations were carried out before the drug administration and subsequently in the fifth week, in the twelfth week and at the end of the experiment.

The *number of erythrocytes* was determined as usual in the Bürker chamber The *number of reticulocytes* was determined in the Ehrlich counting ocular by the Schudel technique. The *haemoglobin concentration* of the blood was determined by means of the Zeiss haemometer Some of the results were checked by a photometric procedure (oxyhaemoglobin method, Hg filter 578 mμ) Generally the *methaemoglobin* (haemiglobin) *concentration* was determined by the method of KIESE (1947) the haemiglobin is reduced by addition of Na-hypodisulphite, converted to COHb by introduction of CO and determined photometrically With difference measurement a calibration curve is not required the findings have been given as extinction values. We also tried to apply the cyanide micromethod of Evelyn and Malloy (WILKINSON 1960) but have found it to involve even greater methodological error than the CO method, and we therefore did not use it in further studies The *creatinine content* of the serum was determined by the photometric method of POPPERL *et al* on the basis of the directions for use of the photometer Eppendorf" The prothrombin time was determined by means of the "micromethod Geigy which is a modification of the Quick method. The coagulation time was not calculated, since only relative changes in prothrombin time were of interest The *specific gravity of urine* was determined by means of aerometers, and the urinary albumin was determined on an arbitrary scale with sulphosalicylic acid At the beginning of the investigation the urine was collected in metabolism chambers. Later catheter specimens were drawn off under Pernocton ® (β-Bromallyl-butylbarbituric acid-Na) narcosis (40 mg/kg intravenous) from male and female animals, by which means they could be obtained readily and conveniently

Results

During the first four weeks of the chronic toxicity test daily doses of 25 mg/kg of the test preparations were administered Changes in the clinical picture and in the behaviour of the test animals were not observed during this period. It can be seen from table I that there were no substantial changes in haematological and other findings. Eight weeks after the daily dose had been increased to 50 mg/kg daily a repetition of these determinations did not show any effect on the animals in any of the three groups, and the experiment was therefore continued for a further 14 weeks.

During the entire test period the animals showed no changes in normal behaviour Further there were no sensory changes There was initially a steady increase in weight, but there were differences between the groups

Table I

Clinical Observations on Cats (Mean Values of the Groups).

	Group	Clinical Examination: at the beginning	after 4 weeks	after 11 weeks	at the end
Erythrocytes (mill./mm³)	A	5.72	6.08	6.74	7.70
	P	6.37	6.16	6.32	5.70
	N	5.99	5.94	7.05	6.50
Reticulocytes (%)	A	3.0	3.1	1.3	0.4
	P	3.6	3.0	1.3	1.3
	N	2.8	2.9	1.2	1.6
Haemoglobin (g/100 cc.)	A	10.5	11.8	13.0	13.1
	P	12.3	11.7	11.9	9.1
	N	11.8	11.6	12.4	10.9
Met-Hb (E)	A	0.043	0.029	0.009	0.009 [1])
	P	0.035	0.033	0.012	0.005 [1])
	N	0.011	0.037	0.018	0.006[1])
Creatinine (mg %)	A	2.30	1.86	0.87	1.08
	P	2.38	1.92	1.23	1.21
	N	2.23	1.72	1.02	1.07
Prothrombin Time (sec.)	A	11.1	11.0	15.1	10.7
	P	11.6	12.1	13.8	10.7
	N	12.0	12.3	14.1	11.8
Urine Specific Gravity	A	1018	1013	1023	1027
	P	1018	1014	1025	1027
	N	1023	1014	1027	1026
Urinary Protein[2])	A	3 Ø, 3	1 Ø 3? 2+	1 Ø 1?, 4+	4+
	P	6 Ø	4 Ø 2?	1? 4+	1 Ø 2+
	N	3 Ø 3?	3 Ø 2? 1+	2 3+	1 Ø 2?

[1]) Mean values of two examinations.
[2]) Sample negative Ø, positive +, questionable ?

At about the 23rd test week almost all animals in the NAPA group showed a decreased weight due to loss of appetite. Up to the end of the test period they took less food than the animals in the two other groups.

During the experiment altogether 11 cats died, four of group A, three of group P and four of group N. The first deaths occurred in the 12th week, when one animal of each group died (A 6, P 4, N 2). P 4 probably died from shock during the withdrawal of blood, the other two animals died one day later. It is possible that these deaths were also connected with the withdrawal of blood. The autopsy findings provided no further indications. In the 18th week the cats A 4 and P 2 died. Autopsy showed that cat A 4 probably died from pneumonia. Cat P 2 died after loss in weight had been observed in it during the preceding days.

Cat N 1 died in the 24th week Apart from otherwise normal autopsy findings, the liver showed strong marmoration In the 27th week cat N 4 died and further deaths occurred in the 28th (P 1) and the last week (A 1 A 5 and N 5) The gross pathological findings on N 1 N 4 and P 1 would allow the conclusion that there is a relationship between the medication and death, but it is legitimate to assume that the three animals dying during the last week had been weakened by the withdrawal of blood during the previous experimental period

The results of the haematological and other clinical tests are shown in table 1

In the course of the experiment the number of erythrocytes of the animals in groups A and N increased the animals of the phenacetin group on the other hand showed a decrease.

In accordance with the changes in the number of erythrocytes, an increase in the haemoglobin content was observed in the cats of the acetylsalicylic acid group whereas the haemoglobin content decreased in those of the phenacetin group The animals of group N did not show any changes.

In all three groups the number of reticulocytes decreased to half the initial values or even lower in the course of the experiment. According to SCHERMER (1954) this may be explained by the fact that young cats have far more reticulocytes than older animals As normal values SCHERMER indicates 2 to 3‰. Thus the values obtained in the last examinations, especially those for group A were below normal.

The creatinine content of the serum of all groups showed a steady decrease during the experiment.

In all three groups the methaemoglobin values decreased during the experiment. The reverse reaction would have been expected at least in the phenacetin group Since repeated determinations furnished the same values, the comparatively low methaemoglobin values towards the end of the experiment must be regarded as correct.

No influence of any of the three test substances on the prothrombin time was observed

In the course of the experiment the specific gravity of the urine of all animals increased slightly but this finding is of little importance, since all values are within the normal range (1020 to 1040) In all three groups slight proteinuria was observed The increase in specific gravity was probably attributed to the protein content of the urine

The animals that died in the course of the experiment and those killed at the end of the experiment by intravenous injection of a barbiturate were subjected to post mortem examination However in some of the cats that had died during the experiment (A 4 A 6, P 2, and N 2) a complete

autopsy was not considered advisable, since 12 or more hours had elapsed between the time of death and its discovery and autolytic changes had therefore taken place.

After the autopsy samples were taken from liver kidneys, lungs, spleen and suprarenals, and from the bladders of two animals. The histopathological findings in those livers and kidneys suitable for examination are summarized in table 2. The results of the macroscopic examinations are not shown, since they did not furnish any useful information.

The most important result of the comparative histopathological findings is that in groups P and N fatty degeneration of the liver and even necrotic changes occurred, whereas in group A (acetylsalicylic acid) a moderate fatty degeneration was observed only in one of five animals. Three or four animals of the phenacetin group showed fatty degeneration and one necroses of the liver and three of the six animals of the NAPA group showed moderate to severe fatty degeneration of the liver and two of them even severe necrotic changes. In the two last-mentioned animals an affection of the liver parenchyma could be regarded as the cause of death.

The pathological changes in kidneys and the other organs were found in all test groups. There is no evidence of any systematic influence exerted by the three test substances on the histological picture.

Discussion

Several authors have reported that formation of methaemoglobin was observed in man and animals after the administration of phenacetin (LESTER 1943 GREENBERG and LESTER 1946 BOREUS & SANDBERG 1954 ENGELHARDT *et al.* 1959). However SCHAUB *et al.* (1953) failed to produce pathological blood pigments in rabbits with phenacetin. According to LESTER, cats are twice as sensitive as man to changes in blood pigments caused by phenacetin and, according to STAUB (1958) they are therefore particularly suitable for toxicological examinations. Nevertheless, we did not observe any increase in the formation of methaemoglobin after administering substantial quantities of phenacetin to cats for several months. The methaemoglobin concentrations did not differ from those of animals given NAPA or acetylsalicylic acid. These findings agree with those obtained by PLETSCHER *et al* (1958) who did not observe any difference between phenacetin and NAPA in their effects on the life-time of erythrocytes.

Further interstitial nephritis was not observed by us in any animal. Chronic tests on other species of animals showed similar results (*e g* STUDER & ZBINDEN 1955 PLETSCHER *et al.* 1958 MIESCHER & STUDER 1961). Only THÖLEN *et al.* (1956) found evidence for the beginning of interstitial

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nephritis after applying to mice a preparation containing phenacetin. Recently ESKALDO & TALANTI (1961) reported nephritic changes in kidneys of rats, which they detected by histochemical methods. Similar changes were also observed on administering phenacetin or NAPA. The results obtained in our experiment on cats again show that there appear to be species differences and that the results of animal experiments are of minor importance for and discussion of the relationship between the abuse of phenacetin and interstitial nephritis in man.

Yet the histopathological findings on the livers deserve special attention. In general, phenacetin, and especially NAPA, appeared to be more toxic than acetylsalicylic acid if administered to cats. Nevertheless, owing to the small number of animals used in the experiment unequivocal conclusions cannot be drawn. It is not yet possible to discuss validly the mechanism of this affection.

Apart from these results, the toxicity study has again shown the general difficulties that have to be expected from the use of cats in chronic toxicity tests.

Summary

1 On chronic oral administration to cats, N-acetyl-p-aminophenol (NAPA) caused severe affections of the liver Phenacetin administered in the same doses also caused affections of the liver although of less severity In general, NAPA appeared to be more toxic than phenacetin in cats.

2 Histopathological findings did not show distinct changes in the kidneys caused by phenacetin, NAPA or acetylsalicylic acid. Haematological findings also especially the methaemoglobin contents were not markedly affected in cats by any of the three substances.

3 On chronic administration to cats the same amount of acetylsalicylic acid is apparently of lower general toxicity than phenacetin or NAPA.

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Table 2

Summary of Histopathological Findings.

Group	Animal	Liver	Kidneys
A	1)	Light acute congestion	Light cloudiness of the tubulus epithelium. Some glomeruli. Swelling
	2	No pathological changes	No pathological changes
	3	Moderate fatty degeneration	Single hyaline changes, cloudy swelling of the tubulus epithelium
	4)	No pathological changes	–
	5)	Moderate acute and chronic congestion	No pathological changes
	6)	–	–
P	1)	Acute congestion. Fatty degeneration of parenchyma. Focal necrosis	Dilatation of tubuli, hyalinecasts in the tubuli
	2)	–	–
	3)	Light fatty degeneration	–
	4)	–	No pathological changes
	5	Moderate acute congestion	No pathological changes
	6	Marked fatty degeneration	No pathological changes
N	1)	Acute congestion, marked fatty degeneration, sporadic severe necroses	Pyelonephritis, cortical oedema
	2)	Moderate fatty degeneration	
	3	Acute congestion, moderate fatty degeneration, lymphocytic infiltrations	Marked cloudy swelling, dilatation of tubuli
	4)	Early cirrhoses, severe fatty degeneration, severe necroses	–
	5)	Moderate acute congestion, deposition of pigments	No pathological changes
	6	Acute congestion	No pathological changes

) Died during the test period

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Erratum

In *Acta pharmacol. et toxicol.*, 1963, 20, p. 373, line 7 f. b., *for* "preganglionic" *read* "postganglionic".

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From the Research Laboratories of AB Astra, Södertälje, Sweden.

Catechol-O-Methyl Transferase Inhibitors. *In Vitro* Inhibition of the Enzyme in Mouse-Brain Extract

By

S. B. Ross and Ö. Haljasmaa

(Received May 16, 1964)

Favourable findings of the therapeutic effect of some inhibitors of monoamine oxidase (MAO) have prompted a search for inhibitors of other enzymatic pathways for the inactivation of catecholamines. For the inactivation of circulating and exogenously supplied catecholamines catechol-O-methyl transferase (COMT) is of great significance (AXELROD, WEIL-MALHERBE & TOMCHICK 1959), and this enzyme also seems to be responsible for the enzymatic catabolism of noradrenaline at sympathetic nerve endings, whereas MAO inactivates catecholamines leaking out from storage granules (AXELROD & HERTTING 1961, BRODIE & COSTA 1962).

In the investigation to be reported below several classes of compounds were studied for their capacity to inhibit COMT *in vitro*.

Methods

COMT activity *in vitro* was determined by the method of AXELROD, ALBERS & CLEMENTS (1959) with minor modifications (ROSS 1963). Mouse brains were homogenized in one volume of isotonic KCl solution with MSE homogenizer. A measured portion of the supernatant from the centrifuged homogenate (14.000 g for 20 min. at 0° C) was incubated at 37°C for 90 min. with 2.8 mμmol DL-noradrenaline-7-^{3}H (260 mμc) 2 μmol $MgCl_2$, 20 mμmol cysteine hydrochloride, 80 mμmol S-adenosyl-methionine iodide (Sigma, several preparations were used), 28 μmol phosphate buffer (pH 7.8) and the compound to be tested. The final volume was 0.5 ml. After addition of 1 ml of M/2 borate buffer (pH 10) and 3 g of solid NaCl, the normetanephrine formed was extracted with 15 ml of a mixture of toluene and isoamylalcohol (3:2). The radioactivity in 5 ml of the organic phase was determined by liquid scintillation counting (Packard TriCarb).

The inhibitory effect of the various substances on COMT was compared with that of pyrogallol, which was used as a standard in each determination. At least three different concentrations of the compounds and the standard were tested in duplicate

Table 1

Inhibition of COMT by catechol derivatives in vitro. The enzyme activity was determined in mouse brain extract. Substrate, 5.6 mμmol/ml noradrenaline-^3H. Incubation time, 90 min. at 37°C. The inhibition of COMT activities was determined at 50 per cent inhibition level and compared with that of pyrogallol, used as standard inhibitor

Compound	C_3	C_4	C_5	C_6	50 per cent inhibition of COMT mμmol/ml	COMT inhibiting potency (pyrogallol = 1)
Catechol	H	H	H	H	32	0.07
Pyrogallol	OH	H	H	H	~1	1.0
Hydroxyhydroquinone	H	OH	H	H	295	0.007
Tetrachlorocatechol	Cl	Cl	Cl	Cl	2.5	0.8
Tetrabromocatechol	Br	Br	Br	Br	4.0	0.5
3-Methoxycatechol	OCH_3	H	H	H	2.6	0.8
3-Methylcatechol	CH	H	H	H	16	0.13
H 17/77	CH_2CONH_2	H	H	H	>1200	<0.002
H 13/49	H	CH_2CONH_2	H	H	3.5	0.6
H 17/57	H	CH_2CONH_2	Br	H	12	0.18
H 29/55	CH(C_2H)-CONH	H	H	H	>1000	<0.002
Protocatechualdehyde	H	CHO	H	H	2.8	0.8
Protocatechuic acid	H	COOH	H	H	210	0.01
2,3-Dihydroxybenzoic acid	COOH	H	H	H	>1300	<0.002

Catechols with nuclear substitution

Substitution of the catechol molecule considerably changed the COMT inhibitory capacity (table 1). Thus, pyrogallol had ten times the potency of catechol, whereas hydroxyhydroquinone had only one hundredth of the inhibitory action of pyrogallol. The carboxyl group in 3-position (2,3-dihydroxybenzoic acid) eliminated, and in 4-position strongly reduced, the inhibitory effect protocatechuic acid had one hundredth of the potency of pyrogallol. Protocatechualdehyde and 3-methoxycatechol were almost as potent as pyrogallol. Substitution of the catechol by carboxamidomethyl in the 3-position (H17/77) eliminated the inhibitory effect, whereas the compound with the carboxamidomethyl group in the 4-position (H 13/49) was still about half as active as pyrogallol.

Some 4-substituted catechols.

Since catecholamines are the natural substrates for COMT several 4-substituted catechol derivatives were studied for their capacity to inhibit the enzyme. Table 2 shows that 3 4-dihydroxyphenyl-substituted carboxylic acids were poor inhibitors of the enzymic methylation of noradrena-

or triplicate. The concentration of the compound resulting in a 50 per cent inhibition of the COMT activity was determined graphically

The Michaelis constant for the enzyme reaction with noradrenaline as substrate was determined by the method of LINEWEAVER & BURK (1934) The substrate concentrations in these experiments were 2.8 5 6 11.2 and 22.4 mμmol per ml and the incubation time 30 min

Results

The product formed and extracted under the conditions used was tested by paper chromatography with n buthanol acetic acid and water (8 2 2) as solvents It had the same R_f value as normetanephrine. In fig. 1 the enzyme activity obtained has been plotted as a function of time, and it appears that during the first 30 minutes the relation was almost linear At a substrate concentration of 5 6 mμmol/ml the activity of the enzyme obtained from 1 g brain was about 20 mμmol normetanephrine per hour With the S adenosylmethionine concentration used the Michaelis constant was 2×10^{-5}M This value is higher than that found by AXELROD, ALBERS & CLEMENTE (1959) with adrenaline as substrate and rat brain extract as enzyme, but it is considerably lower than that found by CROUT (1961) with noradrenaline as substrate and rat brain extract as enzyme.

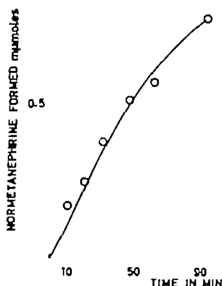


Fig. 1 Time curve for O-methylation of noradrenaline by mouse brain extract. Noradrenaline concentration 5 6 mμmol/ml.

Table 3

Inhibition of COMT by some 3 4-dihydroxyphenylethylamine derivatives.
Experimental conditions as for table 1

Compound	HO, HO–⟨ring⟩–C – CH N: C_β	C_β	C_α	N	N	N	50 per cent inhibition of COMT mμmol/ml	CONT inhibiting potency (pyrogallol = 1.0)
Dopamine.	H	H	H	H	H	H	6.3	0.3
Noradrenaline.	H	OH	H	H	H	H	42	0.05
L 3,4-Dihydroxynorephedrine	H	OH	CH	H	H	H	42	0.05
-Adrenaline	H	OH	H	CH_3	H	H	13	0.2
DL N-Trimethylnoradrenaline	H	OH	H	CH_3	CH_3	CH_3	16	0.1
DL-Isoproterenol	H	OH	H	C H_7-iso	H	H	6.3	0.3
Adrenalone	O		H	CH	H	H	9 0	0..

Some pyrogallol derivatives.

Substitution of pyrogallol affected the COMT inhibitory capacity in the same way as substitution of catechol (table 4). Thus, *e g* gallic acid was a poor inhibitor On the other hand, the esters of gallic acid were even stronger inhibitors than pyrogallol. 2,3 4-Trihydroxybenzoic acid had about 30 times the potency of gallic acid, and 2,3 4-trihydroxyacetophenone was as potent as pyrogallol. Gallamide had about $^1/_{100}$ and 3 4,5-trihydroxyphenylacetamide about $^1/_{25}$ the activity of pyrogallol

Table 4

Inhibition of COMT by some pyrogallol derivatives.
Experimental conditions as for table 1

Compound	HO, OH, OH ring: C_5	C_6	50 per cent inhibition of COMT mμmol/ml	COMT inhibiting potency (pyrogallol = 1.0)
Gallic acid	COOH	H	400	0 005
2,3 4-Trihydroxybenzoic acid	H	COOH	12	0.2
Gallamide	CONH	H	150	0.01
3 4,5-Trihydroxyphenylacetamide	CH_2CONH	H	39	0.04
Methyl ester of gallic acid	COOCH	H	1.0	2.1
Isopropyl ester of gallic acid	$COOC_3H$ iso	H	1 4	1.5
2,3 4-Trihydroxyacetophenone	H	$COCH_3$	2.1	1.0

Table 2

Inhibition of COMT by some 3 4-dihydroxyphenyl derivatives. Experimental conditions as for table 1

Compound	HO OH (ring) C$_4$	C$_6$	50 per cent inhibition of COMT mμmol/ml	COMT inhibiting potency (pyrogallol = 1.0)
3 4-Dihydroxyphenyl acetic acid	CH_2COOH	H	~80	0.008
Hydrocaffeic acid	CH_2CH_2COOH	H	75	0.03
Caffeic acid	$CH = CHCOOH$	H	29	0.07
L DOPA	$CH_2CH(NH_2)COOH$	H	7 5	0.3
D-DOPA	$CH_2CH(NH_2)COOH$	H	>1000	<0.002
L-α Methyl-DOPA	$CH_2C(CH_3)(NH_2)COOH$	H	>950	<0.002
Esculetin	O-COCH = CH		2.6	0.8
4-Methylesculetin	O-CO CH = $C(CH_3)$-		1 8	1.2
5 6-Dihydroxyindole	CH = CH NH		260	0.008
H 17/53	$CH(OH)CONH_2$	H	1 6	1.3
H 17/87	$CH(OCH_3)CONH_2$	H	2.1	1.0
H 22/07	$CH(OC_2H_5)CONH_2$	H	6.7	0.3
H 22/21	$CH(CH_3)CONH_2$	H	6.7	0.3
H 22/41	$CH(C_2H_5)CONH_2$	H	8.1	0.3
H 22/44	$C(CH_3)_2CONH_2$	H	3 0	0.07
H 22/59	$CH(C_3H_7\text{-iso})CONH_2$	H	9 3	0.2
H 22/54	$CH(C_3H_7\text{-n})CONH_2$	H	15	0.1
H 22/58	$CH(C_4H_9\text{-n})CONH_2$	H	9 6	0.2
H 22/98	$CH(C_4H_9\text{-iso})CONH_2$	H	3 0	0.7
H 22/93	$CH(C_6H_{11})CONH_2$	H	2.6	0.8
H 17/47	$CH = CHCONH_2$	H	4 0	0.5
H 13/48	$CH_2CH_2CONH_2$	H	2.1	1.0
H 22/85	OCH_2CONH_2	H	33	0.07
H 22/57	CH_2CN	H	4 4	0.5
H 17/50	$CH_2CONHNH_2$	H	6.5	0.3

line L DOPA on the other hand was a good inhibitor D-DOPA like L-α methyl DOPA, had no inhibitory power at the concentrations tested. Several amides were considerably more active than the corresponding carboxylic acids, most of them, even more active than catechol The most potent was 3 4-dihydroxymandelamide (H 17/53) Esculetin was almost as potent as pyrogallol 5 6-Dihydroxyindole on the other hand was a poor inhibitor of COMT activity

The inhibition of the enzymatic O-methylation of tritiated noradrenaline by various catecholamines is shown in table 3 Isoproterenol adrenaline and dopamine were more active than non radioactive noradrenaline and 3 4-dihydroxynorephedrine To obtain 50 per cent inhibition of the methylation of tritiated noradrenaline by inactive noradrenaline eight times the normal substrate concentration was required, indicating that the substrate concentration used was far from the optimal

Table 7

Inhibition of COMT by some 8-hydroxyquinoline derivatives. Experimental conditions as for table 1

Compound	C	OH C_5	C	50 per cent inhibution of COMT mμmol/ml	COMT inhibiting potency (pyrogallol = 1.0)
	R	R	R_3		
8-Hydroxyquinoline	H	H	H	1 4	1.5
5,7 Dibromo-8-hydroxy-quinoline.	Br	Br	H	13	0.2
8-Hydroxyquinaldine	H	H	CH_3	1250	0 002

tropolone, 4-isopropyltropolone (γ-thujaplicin) and 4-tropoloneacetamide were about as potent as pyrogallol under the conditions used

8-Hydroxyquinoline derivatives

Since all the active COMT inhibitors mentioned above have metal chelating properties, it seemed appropriate also to test some other metal chelators of various structures. 8-Hydroxyquinoline was 50 per cent more potent than pyrogallol as a COMT inhibitor (table 7). 5 7 Dibromo-8-hydroxyquinoline was only one fifth as potent as pyrogallol whereas 8-hydroxyquinaldine had no COMT inhibitory properties at the concentrations tested.

Compounds with no COMT inhibitory capacities

Several known metal chelators were tested and found to be inactive as COMT inhibitors, *e g* $EDTANa_2$ α,α'-dipyridyl, o-phenanthroline, L-hydroxyantraquinone, phthiocol, juglone, o-pyridone and o-hydroxyacetophenone All these compounds were tested at concentrations about a hundred times higher than that of the substrate. The concentration of magnesium chloride used was nearly ten times higher than that of the test compounds, and the chelation of this metal ion was therefore far from complete in these experiments.

D Iorio & Mavrides (1962) have reported that 3,5-diiodo-4-hydroxy benzoic acid is a COMT inhibitor *in vitro* We found no inhibitory effect of this compound when tested at a concentration one hundred times higher than that of the substrate. At this concentration 3 5-diiodotyrosine also was inactive as a COMT inhibitor

Table 5

Inhibition of COMT by some o-dihydroxy substituted flavonoids. Experimental conditions as for table 1

Compound	R_1 (C_7)	R_2 (C_4)	$-C_3-C_2<$ = $R_3-R_4<$	I50 per cent inhibition of COMT mμmol/ml	COMT inhibiting potency (pyrogallol = 1.0)
D-Catechin	OH	H	CH(OH)-CH<	1 6	1.3
Dihydroquercetin	OH	O	CH(OH)-CH<	2.1	1.0
Quercetin	OH	O	-C(OH) = C<	10	0.2
Rhamnetin	OCH_3	O	C(OH) = C<	7 0	0.3
Cyanidin	OH	H	= C(OH)-C<	42	0.05

o-*Dihydroxyflavonoids*

Axelrod & Laroche (1959) have discussed the possibility that the catecholamine potentiating action of o-dihydroxysubstituted flavonoids may be caused by the inhibitory effect on COMT of these compounds. As shown in table 5 D-catechin was a potent inhibitor of enzymatic O-methylation of noradrenaline, being even more active than pyrogallol. Dihydroquercetin was as potent as pyrogallol quercetin and rhamnetin had about one fifth of the pyrogallol activity Cyanidin was still less potent.

Some tropolone derivatives

Belleau & Burba (1961 1963) have shown that 4-methyltropolone and some other tropolones are potent inhibitors of COMT *in vitro* and *in vivo* A few tropolone derivatives were therefore studied for their COMT inhibitory action by the method used. Table 6 shows that 4-methyl-

Table 6

Inhibition of COMT by some tropolone derivatives. Experimental conditions as for table 1

Compound	C_4	50 per cent inhibition of COMT mμmol/ml	COMT inhibiting potency (pyrogallol 1 0)
4-Methyltropolone	CH_3	2.1	1 0
4-Isopropyltropolone	$CH(CH_3)_2$	2 6	0.8
4-Tropoloneacetamide	CH_2CONH_2	3 2	0.7

flavonoids. By conjugation of the ethylene group with the catechol residue of the molecules of quercetin, rhamnetin and cyanidin, these compounds are orientated in a planar manner. In D-catechin and dihydroquercetin, which lack this ethylene group, the catechol part of the molecule can rotate more freely to other planes.

The COMT inhibitory effect of 8-hydroxyquinoline does not seem to have been reported before. This compound is known to be a strong metal chelator, but it is evident that this property is not in itself sufficient to produce COMT inhibition, since several other known metal chelators lacked inhibitory effect. Thus, 8-hydroxyquinaldine had none; it causes precipitation with several metal ions but not with Al^{+++} and with Mg^{++} except at pH values above 8 (MERRIT & WALKER 1944). No soluble complex between 8-hydroxyquinaldine and Mg^{++} seems to be formed at pH 7.8, since no change in ultraviolet absorption of 8-hydroxyquinaldine was obtained in presence of magnesium chloride at this pH (unpublished observation). These results seem to support the concept that Mg^{++} takes an active part in binding the substrate to the enzyme and the methyl donator as proposed by SENOH *et al.* (1959). SENOH, TOKUYAMA & WITKOP (1962) have demonstrated that EDTA inhibited the activity of purified COMT in the absence of Mg^{++}. In our experiments EDTA at a concentration one tenth that of Mg^{++} had no inhibiting effect on COMT. It may be that EDTA chelates Mg^{++} in the solution more readily than it reacts with the metal-containing COMT enzyme. However, besides chelating properties all the inhibitors of COMT so far known have ligands with positions similar to those of the hydroxyls in the catechol molecule. This property may be of great significance for the COMT inhibitory potency of a compound.

Summary

Compounds of several classes were tested for their inhibitory effects on COMT activity in extract from mouse brain *in vitro* with tritiated noradrenaline as substrate.

The most potent inhibitors were esters of gallic acid, D-catechin, 4-methylesculetin, 3,4-dihydroxymandelamide, 8-hydroxyquinoline and 4-methyltropolone, which were as active as pyrogallol or even more active.

Compounds with low inhibiting activity were gallic acid, gallamide, 3,4-dihydroxyphenyl-substituted carbonic acids and 5,6-dihydroxyindole.

The compounds that had no COMT-inhibiting activity at the concentrations tested were 2,3-dihydroxy benzoic acid, 2,3-dihydroxyphenylacetamide, D-DOPA, L-α-methyl-DOPA, 8-hydroxyquinaldine, meconic acid and 3,5-diiodo-4-hydroxy benzoic acid. Several metal chelating agents also had no COMT-inhibiting effect, e.g. $EDTANa_2$.

Discussion

AXELROD & THOMCHICK (1958) have shown that several catechol derivatives are good substrates for COMT *in vitro* Several of the compounds tested probably inhibit the O-methylation of noradrenaline by this substrate activity of their own Presumably compounds that are good substrates for COMT should also be good inhibitors of the enzymatic O-methylation of noradrenaline However the relationship between inhibitory capacity and substrate activity of some of the compounds tested does not seem to support this assumption. According to AXELROD & TOMCHICK (1958) protocatechuic acid and catechol are better substrates for purified COMT from rat liver than catecholamines In our tests protocatechuic acid had only one tenth the inhibitory action of catechol on the noradrenaline methylation and was also less active than the catecholamines tested. The differences between substrate activity and inhibitory capacity may at least in part be explained by the different enzyme sources used

SENOH *et al* (1959) have proposed that the most nucleophilic hydroxyl under the conditions used is methylated by COMT The nucleophilic nature of the hydroxyls depends on changes in the electron distribution induced by various substituents of the catechol molecule. Catecholamines, *e g* have been shown to be 90 per cent methylated in the meta hydroxyl, whereas 3 4-dihydroxyacetophenone is about 50 per cent methylated in the para position (DALY AXELROD & WITKOP 1960) The differences in COMT inhibitory potency of the compounds tested may therefore in part be explained by different affinities to the enzyme, due to changes of the electron distribution in the molecules The poor inhibitory effect of gallic acid as compared with that of 2,3 4-trihydroxybenzoic acid may be an example of this.

The binding of the substrate to the enzyme and S-adenosylmethionine seems to be mediated by a metal complex formation as suggested by SENOH *et al* (1959) That 2,3-dihydroxybenzoic acid and 2,3-dihydroxy phenylacetamide (H 17/77) have no inhibitory effect may be due to the strong hydrogen bond between the carboxyl group and the adjacent hydroxyl in the former compound and between the carbonyl group and the hydroxyl in the latter This hydrogen bond considerably decreases the metal ion binding capacity of the hydroxyls

That D-DOPA had no inhibitory effect is apparently due to steric factors, since L DOPA was a fairly good inhibitor It is to be remembered that L-α methyl-DOPA also had no inhibitory effect on COMT

Steric interference of the compounds with the enzyme complex may also explain the differences in inhibitory actions of the o-dihydroxy substituted

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From the Research Laboratories of AB Astra, Södertälje, Sweden.

Catechol-O-Methyl Transferase Inhibitors. *In Vivo* Inhibition in Mice

By

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(Received May 16, 1964)

Catechol-O-methyl transferase (COMT) seems to be the enzyme principally involved in the catabolism of catecholamines at sympathetic nerve endings (AXELROD & HERTTING 1961), whereas monoamine oxidase (MAO) inactivates catecholamines leaking out from storage granules. Inhibitors of COMT are thus of pharmacological interest, as exerting a potential action on the sympathetic nerve system. So far only few COMT inhibitors have been studied *in vivo* namely pyrogallol, catechol and 4-methyltropolone (AXELROD & LAROCHE 1959 WYLIE, ARCHER & ARNOLD 1960 CROUT 1961a BELLEAU & BURBA 1961 1963 CARLSSON, CORRODI & WALDECK 1963 CARLSSON & WALDECK 1963)

In a previous investigation (ROSS 1963a) a method was developed for rapid determination of COMT inhibition *in vivo* in mice with simultaneous recording of the stimulating or inhibiting effect of the compound tested on adrenergic β-receptors. By this method the COMT-inhibitory effects of compounds of several classes have been investigated, and the results are reported here. The *in vitro* inhibitory effects of these compounds have been studied previously (ROSS & HALJASMAA 1964a).

Methods

COMT inhibition in peripheral organs.

The COMT inhibition in peripheral organs *in vivo* was determined by recording the prolonging effect of the various compounds on tachycardia induced by isoproterenol in anaesthetised mice. The method used has been described in a previous paper (ROSS 1963a). The compounds to be tested were injected *i.p.* 20 minutes before *i.v.* administration of 10 μg/kg

Acknowledgement

We thank Dr H Corrodi, AB Hässle, Göteborg, Sweden, for generous supplies of the compounds marked H in the tables, Dr B Sjöberg, AB Astra, Södertälje, Sweden, for the synthesis of tetrachlorocatechol and tetrabromocatechol and Dr B Carnmalm AB Astra, Södertälje, Sweden, for the synthesis of 5 6-dihydroxyindole

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Results

COMT inhibition in peripheral organs

Of the compounds tested, the tropolones were the most active in prolonging isoproterenol-induced tachycardia in mice (table 1), findings in agreement with the marked COMT inhibition of 4-methyltropolone *in vivo* reported by BELLEAU & BURBA (1961). In our test 4-tropoloneacetamide was slightly more active than the methyl derivative and the isopropyl derivative less active.

ROSS & HALJASMAA (1964a) have shown that 8-hydroxyquinoline inhibits COMT *in vitro*. This compound was found also to inhibit the enzyme to some extent in peripheral organs *in vivo* (table 1).

D IORIO & MAVRIDES (1962) have reported that 3,5-diiodo-4-hydroxybenzoic acid was a COMT inhibitor *in vitro*. That this compound also had some *in vivo* action on this enzyme is indicated by its isoproterenol-potentiating action indicated in table 1.

Pyrogallol had one fifth to one twentieth the activity of the tropolones as a COMT inhibitor in peripheral organs *in vivo* (table 2). The ortho-

Table 1

COMT inhibition, sympathomimetic effect and adrenergic β-receptor blocking action of some non-hydroxyphenyl substituted compounds in mice in vivo

Prolongation of isoproterenol-induced tachycardia was taken as COMT inhibition in peripheral organs. ED300 in mg/kg was the dose of the compound, injected i.p. 20 min. before isoproterenol sulphate, 10 μg/kg causing 3 times the normal duration of the tachycardia.

COMT inhibition in brain was determined by recording the enzyme activity in brain extract from mice pre-treated with the compound 30 min. before decapitation. ED50 in mg/kg was the dose injected i.p. decreasing the enzyme activity by 50 per cent.

Increase of heart rate was taken as due to the sympathomimetic effect of the compound, and the dose noted was the minimal one with a clearly observable effect.

Decrease of the isoproterenol-induced tachycardia was taken as adrenergic β-receptor blocking effect. ED50 in mg/kg was the dose decreasing the isoproterenol-induced increase of the heart rate by 50 per cent.

> highest dose tested without effect. – = effect could not be evaluated. ( ) = not tested.

Compound	COMT inhibition: Peripheral ED300	COMT inhibition: Brain ED50	Sympathomimetic effect: Dose in mg/kg	Adrenergic β-receptor blockade ED50
4-Methyltropolone	10	200	>100	>100
4-Isopropyltropolone	20		>25	>25
4-Tropoloneacetamide (H 17 27)	5	500	>500	>500
8-Hydroxyquinoline	100	100	>100	>100
3,5-Diiodo-4-hydroxybenzoic acid	250	( )	>500	>500

isoproterenol sulphate The heart rate was determined by ECG recordings of 10 sec. at five-minute intervals, and the duration of the tachycardia was defined as the time from the injection of isoproterenol to the moment when the increase in heart rate had slowed down to half its maximum value. The COMT inhibitory potencies of the compounds tested were compared by determining the dose producing a threefold increase in the normal duration of the tachycardia As a rule, three different doses of each compound were injected each dose being tested on three mice.

Sympathomimetic effect and adrenergic β-receptor blocking action

Besides the peripheral COMT inhibitory potencies of the compounds, their sympathomimetic effect on adrenergic β-receptors and their β-receptor blocking power also were determined by the method previously described (Ross 1963a) The compounds were regarded as having a sympathomimetic action of their own when they caused a distinct increase in heart rate and the dose necessary to produce such an increase was determined A reduction of the increase in heart rate induced by isoproterenol was judged as a β-receptor blocking effect and the dose reducing the isoproterenol response by about 50 per cent was estimated

Reduction of COMT activity in mouse brain

The inhibition of COMT activity in mouse brain *in vivo* induced by a compound was determined by estimating the COMT activity *in vitro* of centrifuged brain extracts of mice that had been pre-treated by its *i.p.* injection. Unless otherwise stated the mice were killed half an hour after the injection The brains of four mice were pooled and homogenised in an equal volumes of ice-cold isotonic KCl The homogenate was centrifuged at 14000 g for 20 minutes at 0 C The COMT activity of the extract was determined by the method of AXELROD ALBERS & CLEMENTE (1959) with some minor modifications (Ross 1963a) Tritiated noradrenaline, 2.8 mμmol was used as substrate An aliquot of 0 1 ml of the extract was taken and the final volume was 0 4 ml Each extract was submitted to two or three determinations two separate tests being made for each dose of the compound. The inhibition of COMT activity was expressed as a percentage of the enzyme activity of untreated mice A graphic estimate was made of the dose inhibiting the normal enzyme activity by 50 per cent.

The compounds tested were in part, obtained commercially and, in part synthesized by Dr H Corrodi AB Hässle Göteborg, Sweden (compounds marked H in the tables below) The COMT inhibitory capacities of some of the latter compounds have also been tested by other methods (CARLSSON, CORRODI & WALDECK 1963)

Table 3

COMT inhibition, sympathomimetic effect and adrenergic β-receptor blocking action by o-d hydroxyphenylacetamide derivative in mice.

C mpound	HO OH ring: C_4	C_6	COMT inhibition: Peripheral ED300	COMT inhibition: Brain ED50	Sympathomimetic effect. Dose in mg/kg	Adrenergic β-receptor blockade ED50
H 13/49	-CH_2CONH	H	100	700	>1000	>1000
H 17/53	-$CH(OH)CONH$	H		700	100	100
H 17/87	-$CH(OCH_3)CONH$	H	50	350	>750	>750
H 22/07	-$CH(OC_2H_5)CONH_2$	H	100	350	>1000	1000
H 22/21	-$CH(CH_3)CONH$	H	150	350	>1000	1000
H 22/41	-$CH(C_2H_5)CONH_2$	H	75	250	250	250
H 22/44	-CH(CH)$_2$CONH$_2$	H	150	300	>500	>500
H 22/54	-$CH(C_3H_7)CONH_2$	H	75	400	>500	500
H 22/58	-$CH(C_4H_9)CONH$	H	150	200	>500	500
H 22/59	-$CH(CH(CH_3)_2)CONH$	H	150	250	>150	>150
H 22/93	-CH(C$_4$H)CONH	H	75	200	>100	100
H 22/98	-$CH(CH_2CH(CH_3)_2)CONH_2$	H		( )	>100	100
H 17/77	H	-CH_2CONH	250	( )	>250	250
H 29/55	H	-$CH(C_2H_5)CONH_2$	>250	( )	>250	>250

) See legend to table 1 for explanation

substituted derivatives of pyrogallol vi 2,3 4-trihydroxybenzoic acid and 2,3 4-trihydroxyacetophenone, were slightly more active than pyrogallol Gallic acid, on the other hand was considerably less potent its inhibitory effect has been shown to be insignificant also *in vitro* (Ross & Haljasmaa 1964a) The isopropyl ester of gallic acid had about the same potency as pyrogallol (table 2)

One of the most active compounds of the o-dihydroxyphenols tested was D-catechin whose potency was slightly higher than that of pyrogallol. 3-Methoxycatechol caffeic acid and protocatechualdehyde had the same potency as pyrogallol (table 2)

Several o-dihydroxyphenylacetamide derivatives were studied for their peripheral COMT inhibitory effects (table 3) When the acetamide group was in the ortho position to one of the hydroxyls (H 17/77 and H 29/55), the compound had no inhibitory action These compounds have been shown to lack inhibitory action *in vitro* also (Ross & Haljasmaa 1964a). On the other hand, several of the 3 4-dihydroxyphenylacetamide derivatives, were as potent as pyrogallol H 17/87 H 22/41 H 22/54 and H 22/93 being even slightly more effective judged by their effect on heart rate.

COMT inhibition in brain

As appears from table 1 the tropolones were considerably less potent as COMT inhibitors in brain than they were in peripheral organs. Some

Table 2

COMT inhibition, sympathomimetic effect and adrenergic β-receptor blocking action by o-dihydroxyphenyl derivatives in mice

Compound	COMT inhibition		Sympathomimetic effect Dose in mg/kg	Adrenergic β-receptor blockade ED50
	Peripheral ED300	Brain ED50		
Catechol	50	50	50	50
Pyrogallol	100	125	250	250
3-Methoxycatechol	100	200	250	250
3-Methylcatechol	100	250	100	100
Protocatechualdehyde	100	50	100	100
Protocatechuic acid	200	500	250	250
Caffeic acid	100	250	500	500
Gallic acid	50	500	500	500
2,3 4-Trihydroxybenzoic acid	50	500	200	200
Gallamide		500	250	250
Isopropyl ester of gallic acid	100	100	200	200
2,3 4-Trihydroxyacetophenone	75	100	200	200
Esculetin	( )	350	500	500
4-Methylesculetin		350	100	100
L-α Methyl-DOPA	( )	500	( )	( )
D-Catechin	80	400	100	> 100

) See legend to table 1 for explanation

Nor had 4-tropoloneacetamide any isoproterenol-antagonizing action at even higher doses (table 1)

Duration of the COMT inhibitory effect

The duration of the peripheral inhibitory effects on COMT of some of the most potent substances was also investigated. As judged from the isoproterenol potentiating action (table 4), 40 mg/kg 4-tropoloneacetamide given i.p. had a marked peripheral COMT inhibitory effect 5 hours after injection. H 22/54 500 mg/kg i p., had some COMT inhibitory effect 2 hours after injection. After i.p administration of 200 mg/kg pyrogallol, 3-methoxycatechol, the isopropyl ester of gallic acid, protocatechualdehyde or D-catechin, no COMT inhibitory effect could be observed 2 hours later

The duration of the COMT-inhibitory effect in the brain was also studied with three substances. Table 5 shows that the effect of pyrogallol at a dose of 200 mg/kg i p reached its maximum during the first 30 minutes after the injection and then decreased rather rapidly only one third of the enzyme activity being blocked two hours after the injection. The effect of H 22/54 on the other hand, lasted much longer in brain than in peripheral organs 4 hours after the injection the enzyme activity was still two thirds blocked. The day after the injections the COMT activity was normal. H 13/49 was less long-lasting in effect than H 22/54

Table 4

Duration of the peripheral COMT-inhibitory effect of some COMT-inhibitors. The compounds were injected i.p. at the times noted before administration of isoproterenol sulphate, 10 µg/kg The prolongation of the isoproterenol induced tachycardia was taken as measure of the COMT-inhibitory effect. Each dose was tested on three animals.

Compound	Dose mg/kg	Time before isoproterenol administration, hours	Duration of tachycardia, min.
Control			10
Pyrogallol	200	2	11
Isopropyl ester of gallic acid	200	2	12
2,3,4-Trihydroxyacetophenone	200	2	8
3-Methoxycatechol	200	2	9
D-Catechin	200	2	10
Protocatechualdehyde	100	2	8
H 22/54	200	2	11
	500	2	21
4-Tropoloneacetamide	40	3	31
	40	5	20

of the pyrogallol derivatives, i.e. 2,3 4-trihydroxyacetophenone, the isopropyl ester of gallic acid and also pyrogallol itself were the most potent inhibitors of brain enzyme activity *in vivo* (table 2) The carbon acid derivatives of pyrogallol were less active in brain than in the peripheral organs All the catechol derivatives tested were less effective than the most potent pyrogallol derivatives (tables 2 and 3) While l-α-methyl-DOPA has been shown to have no inhibitory effect on COMT *in vitro* (ROSS & HALJASMAA 1964a) it had some inhibitory action in mouse brain *in vivo* (table 2)

Sympathomimetic effect and adrenergic β-receptor blocking action

One purpose of this investigation was to search for potent COMT inhibitors that have as few side-effects as possible. For this reason the sympathomimetic effects and the adrenergic β-receptor blocking actions of the compounds studied were tested As shown in tables 1–3 several of the compounds reduced the tachycardia induced by isoproterenol this effect was considered to be an adrenergic β-receptor blocking action (ROSS 1963a) Among the compounds causing β-receptor blockade at doses lower than those producing COMT inhibition may be mentioned esculetin 4-methylesculetin gallamide, H 17/53 and H 22/98 At these doses the esculetins and H 17/53 had a sympathomimetic effect as well.

BELLEAU & BURBA (1961) have reported that 4-methyltropolone has adrenergic β-receptor blocking properties and MURNAGHAN & MAZURKIEWICZ (1963) have shown that at high doses it antagonized the lethal effect of adrenaline and the vasodepressor action of isoproterenol in cats. In our test no blockade of the isoproterenol induced tachycardia was found with 4-methyltropolone at doses as high as 100 mg/kg i p (fig. 1).

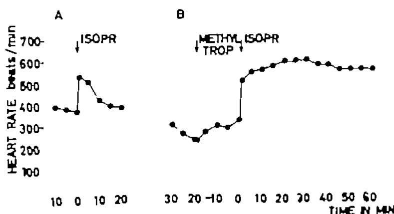


Fig. 1 The action of 4-methyltropolone o isoproterenol induced tachycardia in mice. Means of three determinations. A. Isoproterenol sulphate, 10 μg/kg i. B. 4-Methyltropolone, 100 mg/kg i p. 30 min. before isopr terenol s lphate, 10 μg/kg

mine present in mouse brain *in vivo* the compounds blocked the formation of these metabolites at about the same doses as those producing COMT inhibition in our experiments.

Several of the compounds exhibiting good peripheral effects were much less effective in brain, *viz.* the tropolones, D-catechin and 2,3 4-trihydroxy benzoic acid. The small effect observed in brain may be due to the difficulty of these substances penetrating the blood brain barrier

On the other hand, the COMT-inhibitory effect of L-α-methyl-DOPA in brain is somewhat unexpected, since this compound has been shown to have no similar effect *in vitro* (ROSS & HALJASMAA 1964a) A possible explanation is that the inhibition of COMT *in vivo* was produced by the decarboxylation products of α methyl DOPA, *viz* α-methyldopamin and α-methylnoradrenaline these have been shown to be present in brain after administration of α-methyl-DOPA (CARLSSON & LINDQVIST 1962), and α-methylnoradrenaline is a COMT inhibitor *in vitro* (ROSS & HALJASMAA 1964a).

In contrast to BELLEAU & BURBA (1961) and MURNAGHAN & MAZURKIEWICZ (1963) we found the tropolones tested to have no adrenergic β-receptor blocking action. Since 4-methyltropolone seems to have a papaverine like action (BELLEAU & BURBA 1963), it may be that the reported antagonism of 4-methyltropolone to adrenaline induced toxicity in mice and to the vasodepressor action of isoproterenol in cats is non specific.

Several other compounds tested blocked the adrenergic β-receptors in the same dose range as that causing COMT inhibition. These compounds seem to be unsuitable for studying pharmacological effects when COMT is inhibited. No such sideeffect was found with pyrogallol, the isopropyl ester of gallic acid or 2,3,4-trihydroxyacetophenone, or with several 3,4-dihydroxyphenylacetamide derivatives. These last named compounds, however have been shown to block the enzymatic hydroxylation steps in the biosynthesis of catecholamines and 5-hydroxytryptamine (CARLSSON CORRODI & WALDECK 1963 ROOS & WERDINIUS 1963 ROSS & HALJASMAA 1964b).

Although the tropolones tested seem to be useful for studying the significance of COMT inhibition in peripheral organs *in vivo* they are not suited for evaluation of the role of COMT inhibition in the brain, because of their low potency there. For this purpose, the pyrogallol derivatives seem to be the most suitable COMT inhibitors. Unfortunately these, especially the esters of gallic acid, are comparatively toxic.

Table 5

Duration of the COMT blocking by some COMT inhibitors action in mouse brain *in vivo*

COMT activity was determined in the extract from four pooled brains. Incubation time 90 min. at 37 C. Substrate concentration 2.8 mμmol noradrenaline-7-^{3}H per 0.4 ml. The mice were beheaded at the times noted after i.p injection of the compounds tested. Two independent determinations were made. The COMT activity is expressed as a percentage of that of untreated mice.

Hours after the injection	COMT activity as percentage of normal		
	Pyrogallol 200 mg/kg	H 22/54 500 mg/kg	H 13/49 1000 mg/kg
½	2	16	7
1	21	21	10
2	64	36	16
4	–	33	100
17	–	100	–

Discussion

Izquiredo & Kaumann (1963) have shown that the COMT inhibitor pyrogallol prolongs tachycardia induced in dogs by isoproterenol, whereas this effect is less marked if tachycardia is produced by adrenaline or noradrenaline The same findings have been reported from studies on mice (Ross 1963b) MAO inhibitors had considerably less action on the duration of isoproterenol induced tachycardia in mice (Ross 1963a). The results obtained by recording the prolonging effect of various compounds on tachycardia induced by isoproterenol in mice agree well with the COMT inhibitory effects of these compounds *in vitro* and also with the results obtained by other methods of determining COMT inhibition *in vivo* (Axelrod & Laroche 1959 Carlsson & Waldeck 1963) Thus supports the conclusion that the prolonging effect on isoproterenol induced tachycardia in mice is a good measure of the COMT inhibitory effect of a compound in peripheral organs. It is probable that the effect chiefly involves the enzyme in the liver where the highest activity has been found (Axelrod Albers & Clemente 1959)

Only few investigations have been made into COMT inhibition in brain *in vivo* Crout (1961b) studied the *in vivo* action of pyrogallol on this enzyme in rat brain by a method similar to ours. Our results on mice show good agreement with those obtained by him. Carlsson Corrodi & Waldeck (1963) investigated the action of the 3 4-dihydroxyphenylaceta mide derivatives on the amount of normetanephrine and 3-methoxytyra

mine present in mouse brain *in vivo* the compounds blocked the formation of these metabolites at about the same doses as those producing COMT inhibition in our experiments.

Several of the compounds exhibiting good peripheral effects were much less effective in brain *vivo* the tropolones, D-catechin and 2,3 4-trihydroxybenzoic acid. The small effect observed in brain may be due to the difficulty of these substances penetrating the blood-brain barrier

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Summary

Compounds of several classes were studied for their potencies as inhibitors of COMT activity in peripheral organs and brain in mice *in vivo*

In peripheral organs the tropolones had the highest potency but they were considerably less effective in brain. Pyrogallol the isopropyl ester of gallic acid and 2,3 4-trihydroxyacetophenone were the most effective compounds inhibiting COMT activity *in brain in vivo*

The COMT inhibitory power of several 3 4-dihydroxyphenylacetamide derivatives was the same as that of pyrogallol in peripheral organs, but they were less effective in brain.

Many of the compounds tested interfered with other adrenergic mechanisms, for example stimulation or blockade of adrenergic β-receptors 4-Methyltropolone was found to have no adrenergic β-receptor blocking capacity

For a fairly selective inhibition of COMT in the brain the most effective compounds seem to be pyrogallol and some derivatives of pyrogallol For inhibition of COMT activity in peripheral organs *in vivo* the tropolones seem to be most effective.

Acknowledgement.

For generous supplies of several compounds tested we thank Dr H Corrodi AB Hässle Göteborg, Sweden and for technical assistance Miss Laila Gustafsson and Miss Brita Hägertz.

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Acta pharmacol et toxicol 1964 21 226–239

From the Research Laboratories of AB Astra, Södertälje, Sweden.

Blocking Action of Sympathomimetic Amines on the Uptake of Tritiated Noradrenaline by Mouse Cerebral Cortex Tissues *In Vitro*

By

S. B. Ross and A. L. Renyi

(Received May 29 1964)

The uptake and binding of noradrenaline and adrenaline by tissues are important for inactivation of their biological actions (AXELROD WEIL MALHERBE & TOMCHICK 1959 CROUT 1961) DENGLER, SPIEGEL & TITUS (1961 a, b) have shown that tritiated noradrenaline is actively accumulated by tissues from cerebral cortex, heart and spleen *in vitro* when these tissues are incubated with small amounts of this catecholamine. Sympathomimetic amines, such as tyramine, amphetamine and ephedrine, block the uptake of noradrenaline Other pharmacologically active compounds, *e g* reserpine, cocaine, imipramine, chlorpromazine and dibenzyline, also inhibit this uptake. In a previous study isoproterenol was shown to have but little effect on the uptake of tritiated noradrenaline by mouse heart tissue *in vitro* (ROSS 1963) The powerful action of COMT inhibitors on the duration of the tachycardiac effect of isoproterenol *in vivo* compared with the effects of adrenaline and noradrenaline (IZQUIERDO & KAUMANN 1963 ROSS 1963) may be explained by the slow uptake of the former in tissues. In our investigation several sympathomimetic amines were tested for their blocking effect on the uptake of tritiated noradrenaline by slices from mouse cerebral cortex

Methods

The uptake of tritiated noradrenaline by cortex tissue from mouse brain *in vitro* was determined by the method of DENGLER, SPIEGEL & TITUS (1961 a) About 100 mg of tissue slices were incubated in 2 ml of Krebs-Henseleit solution containing 0.1 mμmol DL-noradrenaline-7-^3H per ml (3 6 C per mmol, New England Nuclear Corp.), and different amounts of the compound were tested in a Dubnoff incubator in an atmosphere of 93 5 / O_2 and 6.5 CO_2 for one hour at 37°C. After incubation the

slices were rapidly wiped on filter paper, weighed and homogenized with 2 ml of absolute ethanol in small centrifuge tubes. A 0.1 ml portion of the incubation medium was added to 2 ml of ethanol. All extracts were centrifuged half an hour later, and the radioactivity in 0.5 ml of the alcohol extract was counted in a liquid scintillation system (Packard TriCarb Autoanalyzator). The scintillation liquid used was 10 ml of a toluene solution of 0.4% 3,4-diphenyloxazole and 0.01% β-bis-/2-(phenyloxazolyl)/-benzene. The ratio of concentration of tritiated noradrenaline in the slices to that in the medium was taken as a measure of the accumulation of noradrenaline in the tissue slices. The percentage blocking effect of the compounds tested was calculated according to the formula

$$\frac{(R_c - R_i)\,100}{R_c - 1}$$

here R_c is the ratio of the control and R_i the ratio in the presence of the inhibitor. At least three different concentrations of the inhibitors in triplicate and controls in triplicate were used. The percentage inhibitions were plotted against logarithms of the concentrations of the inhibitor, and the concentration resulting in 50% inhibition was determined graphically. The slopes of the concentration-response curves were determined as relative figures by dividing the logarithm of the concentration into the value for inhibition.

In several experiments the kinetics of the uptake of tritiated noradrenaline by cerebral cortex tissues were studied. The rates of the uptake reaction were recorded at four different concentrations of noradrenaline (0.025, 0.050, 0.100 and 0.200 mμmol per ml). The incubation time was five minutes, with five minutes pre-incubation at 37°C before the addition of noradrenaline. To obtain a measure of the amount of noradrenaline diffusing into the tissues, some slices were pre-incubated with 30 mμmol cocaine per ml. The difference between the amount of tritiated noradrenaline present in the slices incubated without and with cocaine was taken to be the amount of noradrenaline actively taken up by the tissues. The inverted velocities of the uptake reaction were plotted against the inverted concentrations as described by LINEWEAVER & BURK (1934).

Results

Figure 1 shows the time curve for noradrenaline uptake in cerebral cortex slices at a concentration of 0.1 mμmol per ml. The curve is almost linear during the first 20 minutes, which is in good agreement with the results of DENGLER, SPIEGEL & TITUS (1961 a).

As shown in fig. 2, the rates of uptake of tritiated noradrenaline resembled those of enzymatic reactions according to Michaelis Menden, that is, a linear function was obtained when the inverted velocities were plotted against the inverted concentrations. The Michaelis constant, as determined by the procedure of LINEWEAVER & BURK (1934), was 4×10^{-7}M.

The amount of noradrenaline accumulated in the slices exceeded the concentration in the medium when there was present less than 30 *mμmol* tritiated noradrenaline hydrochloride per ml. The ratio decreased, however, with increasing concentrations. The highest ratio was obtained at a

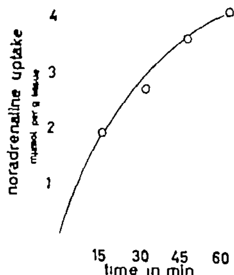


Fig. 1 Uptake of tritiated noradrenaline by mouse cerebral cortex slices as function of time. 0 1 mμmol per ml noradrenaline-³H Krebs Henseleit medium pH 7 4 at 37 C. Means of three determinations

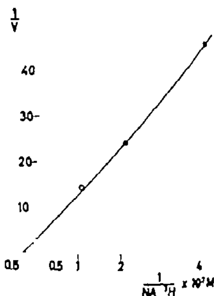


Fig. 2. Rates of uptake of tritiated noradrenaline by mouse cerebral cortex slices at four different noradrenaline concentrations, – 0.2, 0.1 0.05 and 0.025 mμmol per ml. Incubation in 2 ml of Krebs-Henseleit medium at 37 C for 5 min. The inverted uptake velocities (means of three determinations) are plotted against the inverted concentrations.

noradrenaline concentration of 0 025 to 0 1 mμmol per ml (fig. 3) This result is also in good agreement with the findings of Dengler and coworkers.

The blocking action of catecholamines

In a previous investigation noradrenaline and adrenaline were shown to block the uptake of tritiated noradrenaline by mouse heart tissue when added to the incubating medium *in vitro* whereas isoproterenol had only small blocking action (Ross 1963) As shown in fig. 4 the same results were obtained with cerebral cortex tissues Of the catecholamines tested L 3 4-dihydroxynorephedrine was the most potent inhibitor of noradrenaline uptake L Adrenaline was slightly more active than L noradrenaline. Dopamine had only one tenth the activity of noradrenaline (table 1) and the concentration response curve for this compound was steeper than those for the amines mentioned above DL-N Ethylnoradrenaline was less than one tenth as active as L adrenaline, but the slope of the concentration response curves was the same for each DL N Butylnoradrenaline and DL isoproterenol were far less potent than adrenaline or noradrenaline

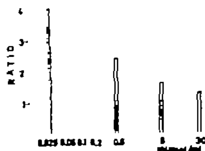


Fig. 3. Ratio of concentration of tritiated noradrenaline in cerebral cortex slices to that in the medium at different noradrenaline concentrations. Incubation in Krebs-Henseleit medium for one hour at 37°C. Means of three determinations at each concentration.

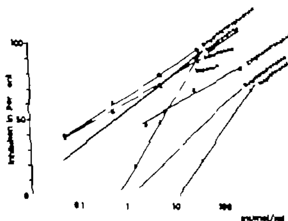


Fig. 4. Inhibition of uptake of tritiated noradrenaline by catecholamines. 0.1 mμmol noradrenaline per ml Krebs-Henseleit medium. Incubation for one hour at 37°C. Means of three determinations at each concentration of catecholamines tested.

3,4-Dihydroxynorephedrine,
Adrenaline, ● L-Noradrenaline,
▲ Dopamine, □ DL-N-Ethylnoradrenaline,
○ DL-N-Butylnoradrenaline, Isoproterenol.

Table 1

Inhibition of uptake of tritiated noradrenaline by sympathomimetic amines.
Experimental conditions as described for fig. 4 The concentration giving 50 per cent inhibition and the slope of the concentration-response curve were determined graphically from fig. 4–7 Nature of receptor-stimulating action according to Trendelenburg (1963)

Amine	Structure C_4 (R_1)	C_3 (R_2)	C_β (R_3)	C_α (R_4)	N (R_5)	Concentration at 50 per cent inhibition mμmol/ml	Slope (inhibition / log. conc.)	Receptor stimulating action	Nature of receptor stimulating action
L-3 4-Dihydroxynorephedrine	OH	OH	OH	CH_3	H	0.19	0 61	α, β	direct
L-Adrenaline	OH	OH	OH	H	CH_3	0.25	0 59	α, β	direct
L-Noradrenaline	OH	OH	OH	H	H	0 57	0 78	α, β	direct
Dopamine	OH	OH	H	H	H	5 7	1 67	α, β	direct
DL N Ethylnoradrenaline	OH	OH	OH	H	C_2H_5	3.2	0 58	β α	direct
DL Isoproterenol	OH	OH	OH	H	C_3H_7	145 0	1 46	β	direct
DL N Butylnoradrenaline	OH	OH	OH	H	C_4H_9	50.0	0 99	β	direct
L 3 Hydroxynorephedrine	H	OH	OH	CH_3	H	0 19	0 57	α, β	direct
L-Phenylephrine	H	OH	OH	H	CH_3	3 6	0 62	α, β	direct
L-Norphenylephrine	H	OH	OH	H	H	10.5	0.83	α, β	direct
DL 3-Hydroxyamphetamine	H	OH	H	CH_3	H	1.9	1 10	α, β	mixed
DL N Ethylnorphenylephrine	H	OH	OH	H	C_2H_5	105.0	1 43	β	direct
L Synephrine	OH	H	OH	H	CH_3	21.0	0 94	α, β	direct
DL Norsynephrine	OH	H	OH	H	H	8 0	1.08	α, β	mixed
DL-4-Hydroxyamphetamine	OH	H	H	CH_3	H	1.3	1 15	α, β	mixed
Tyramine	OH	H	H	H	H	10 5	1.83	α, β	indirect
DL N Butylsynephrine	OH	H	OH	H	C_4H_9	75 0	1.46	β	mixed
DL Norephedrine	H	H	OH	CH_3	H	7 7	0 71	α, β	mixed
DL Amphetamine	H	H	H	CH_3	H	1 9	1 50	α, β	indirect
L-Ephedrine	H	H	OH	CH_3	CH_3	10.5	1 28	α, β	mixed
DL Deoxyephedrine	H	H	H	CH_3	CH_3	4 4	1 39	α, β	
DL Phenylethanolamine	H	H	OH	H	H	27.0	0 93	α, β	mixed
Phenylethylamine	H	H	H	H	H	90.0	2.16	α, β	indirect
N Ethylephedrine	H	H	OH	CH	C H	650 0	1.41	β	

The blocking actions of m- and p-hydroxylated phenylethylamine derivatives

The m-hydroxylated amines were more or equal potent inhibitors of the noradrenaline uptake than the p-hydroxylated (fig. 5 & 6 table 1). L-3-Hydroxynorephedrine was as active as the 3 4-dihydroxy analogue, and these two amines were also the most potent of all amines tested. L-phenylephrine and L-norphenylephrine were considerably less active, but, like 3-hydroxynorephedrine, had the same type of rather flat concentration-response curves as noradrenaline. DL Hydroxyamphetamine was only one third as potent as noradrenaline but had a slightly steeper concentration-response slope than this amine. DL N Ethylnorphenylephrine had low activity as a blocking agent for noradrenaline uptake.

Among the p-hydroxylated amines L synephrine had the least steep concentration-response curve (fig. 6) and had only one fortieth the activity of noradrenaline (table 1) DL-4-Hydroxyamphetamine was the most potent of these amines and had a somewhat steeper concentration-response curve than synephrine. DL Norsynephrine was more potent than synephrine and had a steeper concentration response curve. Tyramine had about the same potency as norsynephrine and an even steeper concentration-response curve. DL N-Butylsynephrine had only a slight inhibitory effect.

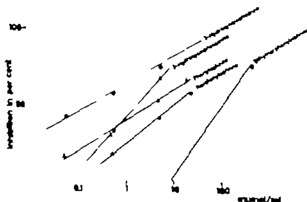


Fig 5 Inhibition of uptake of tritiated noradrenaline by 3-hydroxyphenylethylamine derivatives. Incubation conditions as described for fig. 4.
○ L-3-Hydroxynorephedrine, △ L-Phenylephrine,
● L Norphenylephrine, ✱ DL 3-Hydroxyamphetamine
□ DL N-Ethylnorphenylephrine.

Table 1

Inhibition of uptake of tritiated noradrenaline by sympathomimetic amines.
Experimental conditions as described for fig. 4. The concentration giving 50 per cent inhibition and the slope of the concentration response curve were determined graphically from fig. 4–7. Nature of receptor-stimulating action according to Trendelenburg (1963)

Amine	Structur C_4 (R_1)	C_3 (R_2)	C_β (R_3)	C_α (R_4)	N (R_5)	Concentration at 50 per cent inhibition mμmol/ml	Slope (inhibition / log. conc.)	Receptor stimulating action	Nature of receptor stimulating action
L 3,4-Dihydroxynorephedrine	OH	OH	OH	CH_3	H	0.19	0.61	α, β	direct
L Adrenaline	OH	OH	OH	H	CH_3	0.45	0.59	α, β	direct
L Noradrenaline	OH	OH	OH	H	H	0.57	0.78	α, β	direct
Dopamine	OH	OH	H	H	H	5.7	1.67	α, β	direct
DL N-Ethylnoradrenaline	OH	OH	OH	H	C_2H_5	3.2	0.58	β, α	direct
DL Isoproterenol	OH	OH	OH	H	C_3H_7	145.0	1.46	β	direct
DL N-Butylnoradrenaline	OH	OH	OH	H	C_4H_9	50.0	0.99	β	direct
L 3-Hydroxynorephedrine	H	OH	OH	CH_3	H	0.19	0.57	α, β	direct
L Phenylephrine	H	OH	OH	H	CH_3	3.6	0.62	α, β	direct
L Norphenylephrine	H	OH	OH	H	H	10.5	0.83	α, β	direct
DL 3-Hydroxyamphetamine	H	OH	H	CH_3	H	1.9	1.10	α, β	mixed
DL N-Ethylnorphenylephrine	H	OH	OH	H	C_2H_5	105.0	1.43	β	direct
L Synephrine	OH	H	OH	H	CH_3	21.0	0.94	α, β	direct
DL N-[illegible]ynephrine	OH	H	OH	H	H	8.0	1.08	α, β	mixed
DL 4-Hydroxyamphetamine	OH	H	H	CH_3	H	1.3	1.15	α, β	mixed
Tyramine	OH	H	H	H	H	10.5	1.83	α, β	indirect
DL N-Butylsynephrine	OH	H	OH	H	C_4H_9	75.0	1.46	β	mixed
DL Norephedrine	H	H	OH	CH_3	H	7.7	0.71	α, β	mixed
DL Amphetamine	H	H	H	CH_3	H	1.9	1.50	α, β	indirect
L Ephedrine	H	H	OH	CH	CH_3	10.5	1.28	α, β	mixed
DL Deoxyephedrine	H	H	H	CH	CH_3	4.4	1.39	α, β	
DL Phenylethanolamine	H	H	OH	H	H	27.0	0.93	α, β	mixed
Phenylethylamine	H	H	H	H	H	90.0	2.16	α, β	indirect
D N-Ethylephedrine	H	H	OH	CH	C H	650.0	1.41	β	

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The m-hydroxylated amines were more or equal potent inhibitors of the noradrenaline uptake than the p-hydroxylated (fig. 5 & 6 table 1) L-3-Hydroxynorephedrine was as active as the 3 4-dihydroxy analogue, and these two amines were also the most potent of all amines tested. L-phenylephrine and L-norphenylephrine were considerably less active, but, like 3-hydroxynorephedrine, had the same type of rather flat concentration-response curves as noradrenaline. DL Hydroxyamphetamine was only one third as potent as noradrenaline but had a slightly steeper concentration-response slope than this amine. DL N Ethylnorphenylephrine had low activity as a blocking agent for noradrenaline uptake.

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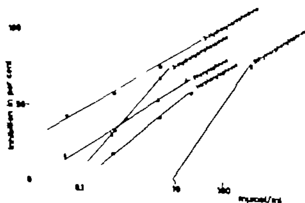


Fig. 5 Inhibition of uptake of tritiated noradrenaline by 3-hydroxyphenylethylamine derivatives. Incubation conditions as described for fig. 4. ○ L 3-Hydroxynorephedrine, △ L-Phenylephrine, ● L Norphenylephrine, ✱ DL 3-Hydroxyamphetamine ☐ DL N-Ethylnorphenylephrine.

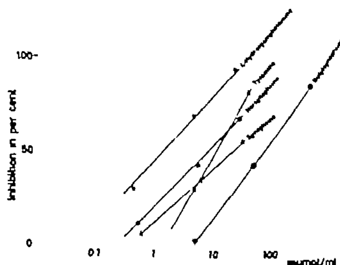


Fig. 6. Inhibition of uptake of tritiated noradrenaline by 4-hydroxyphenylethylamine derivatives. *Incubation conditions as described for fig. 4*
△ L Synephrine, ● DL Norsynephrine,
✱ DL-4-Hydroxyamphetamine, ▲ Tyramine,
○ DL N Butylsynephrine.

The blocking action of non-hydroxylated phenylethylamine derivatives

Fig. 7 and table 1 show that of the phenylethylamine derivatives tested DL norephedrine and DL phenylethanolamine had the least steep concentration response curves. More potent than norephedrine as inhibitors of

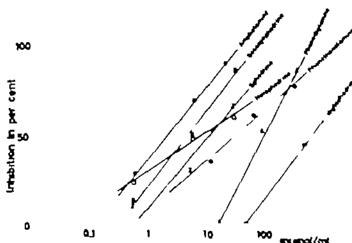


Fig. 7 Inhibition of uptake of tritiated noradrenaline by phenylethylamine derivatives. Incubation conditions as described for fig. 4
○ DL Norephedrine, ● DL Phenylethanolamine,
z Ephedrine, ✱ DL Amphetamine,
ϒ DL Deoxyephedrine, △ Phenylethylamine,
○ DL N-Ethylephedrine.

Table 2.

Inhibition of uptake of tritiated noradrenaline by isomers of some sympathomimetic amines. Experimental conditions as in fig. 4

Amine	Inhibition in per cent		
	5 μg/ml	1 μg/ml	0.1 μg/ml
L Noradrenaline		55	28
DL Noradrenaline		55	28
3-Hydroxynorephedrine	90	75	45
D-3-Hydroxynorephedrine	32	0	0
DL-Amphetamine	73	59	24
D-Amphetamine	85	66	39
Norphenylephrine	67	45	17
DL Norphenylephrine	72	53	31
D-Norphenylephrine	67	50	22

noradrenaline uptake were DL-amphetamine and DL-deoxyephedrine. L Ephedrine was slightly less active than norephedrine, but had a steeper concentration-response curve. Phenylethanolamine was about 50 times less active than noradrenaline, and phenylethylamine was in fact only one third as potent as phenylethanolamine. DL N-Ethylephedrine did not inhibit noradrenaline uptake until concentrations a thousand times higher than that of noradrenaline were reached.

The blocking action of some steroisomers

The inhibitory effects of sterоisomers of some of the amines tested for their uptake of noradrenaline were also studied (table 2). No difference was observed between the L-form of noradrenaline and the racemate, and D-norphenylephrine had the same potency as the L-isomer or the racemate. The L-isomer of 3-hydroxynorephedrine was, however considerably more active than the D-form. D-Amphetamine, on the other hand, was only slightly more active than its racemate.

The effect of MAO inhibition on the blocking action of some sympathomimetic amines

DENGLER *et al.* (1962) have shown that MAO inhibition has no effect on the uptake of tritiated noradrenaline by brain slices. However several

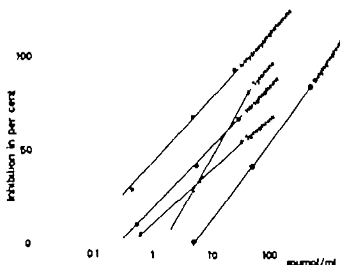


Fig. 6. Inhibition of uptake of tritiated noradrenaline by 4-hydroxyphenylethylamine derivatives. Incubation conditions as described for fig. 4
△ L Synephrine, ● DL Norsynephrine,
✱ DL-4-Hydroxyamphetamine, ▲ Tyramine,
⊙ DL N Butylsynephrine.

The blocking action of non hydroxylated phenylethylamine derivatives

Fig. 7 and table 1 show that of the phenylethylamine derivatives tested DL norephedrine and DL phenylethanolamine had the least steep concentration response curves More potent than norephedrine as inhibitors of

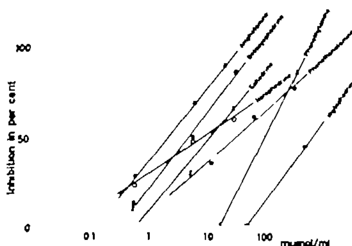


Fig. 7 Inhibition of uptake of tritiated noradrenaline by phenylethylamine derivatives. Incubation conditions as described for fig. 4
○ DL Norephedrine, ● DL Phenylethanolamine
x Ephedrine, ✱ DL Amphetamine,
T DL Deoxyephedrine, △ Phenylethylamine
○ DL N Ethylephedrine.

suggest that the accumulation of tritiated noradrenaline in tissue slices is caused by an active uptake mechanism.

Kinetic experiments determining the initial velocities of the uptake reaction at different concentrations indicate that the reaction mechanism is similar to that of an enzymatic reaction. It may therefore be expressed as



$$NA + C \rightleftharpoons NA \times C \rightarrow NA + C$$

where NA is noradrenaline and C is a hypothetical carrier structure.

The blocking effect on noradrenaline uptake produced by the sympathomimetic amines tested may be caused by different interaction mechanisms.

1) The amines may be taken up by the same mechanism as noradrenaline.

2) The amines may react with the hypothetical carrier structure, but may not be taken up by this mechanism.

3) The amines may not interact with the uptake reaction, but may liberate tritiated noradrenaline already taken up by the tissues.

No differentiation between the mechanisms proposed in 1) and 2) could be achieved in our investigation, since the uptake of sympathomimetic amines tested was not investigated. However non-radioactive noradrenaline must be taken up in the tissues by the same mechanism as the tritiated amine and may inhibit the uptake of the latter in accordance with the first alternative. Adrenaline has been shown to be actively accumulated in brain cerebral cortex tissues *in vitro* (Dengler, Spiegel & Titus 1961 b) and probably blocks the uptake of tritiated noradrenaline by this mechanism. These two amines belong to the group of sympathomimetic amines having the flattest concentration-response curves for inhibition of uptake of tritiated noradrenaline. It is therefore justifiable to suggest that amines with concentration-response curves of this type, e.g. 3,4-dihydroxynorephedrine, 3-hydroxynorephedrine and phenylephrine, inhibit the uptake of tritiated noradrenaline by one and the same mechanism. This assumption is supported by the steep concentration-response curve obtained with dopamine, which has been shown not to be actively taken up by tissue slices *in vitro* (Dengler *et al.* 1962).

The third possibility implies that the amines do not interfere with the uptake mechanism, but liberate the tritiated noradrenaline already taken up. Carlsson, Hillarp & Waldeck (1963) have demonstrated that tyramine is taken up in isolated granules by a different mechanism from those for adrenaline and noradrenaline. The uptake of tyramine by granules requires, in contrast to the uptake of catecholamines, no ATP and Mg^{++}. Tyramine, which has been shown to be a potent catecholamine liberating agent *in vivo* (Bejrablaya, Burn & Walker 1958) and *in vitro* (Schümann & Philippu 1961) also caused liberation of tritiated noradre-

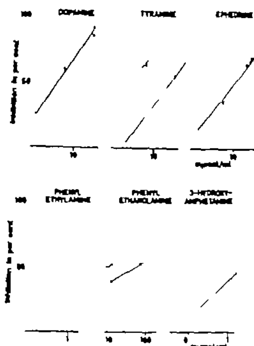


Fig. 8. Effect of MAO inhibition on the blocking action of some sympathomimetic amines against uptake of tritiated noradrenaline. Mice pre treated with 10 mg per kg i.p. pheniprazine hydrochloride four hours before decapitation. Incubation conditions as described for fig 4
solid line no pre-treatment
broken line pheniprazine pre-treatment.

MAO inhibitors blocked the uptake when added to the incubation medium *in vitro* probably by virtue of a sympathomimetic action of their own

Some experiments were performed to study the effect of MAO inhibition on the blockage of noradrenaline uptake caused by some of the amines tested The mice were pre treated with pheniprazine 10 mg per kg i p four hours before decapitation, thus eliminating the sympathomimetic action of this MAO inhibitor Fig. 8 shows the amines that are good substrates for MAO *e g* dopamine tyramine and phenylethanolamine greatly to increase the blocking effect of the noradrenaline uptake when this enzyme was inhibited by pheniprazine However amines known not to be attacked by this enzyme, *e g* ephedrine and 3-hydroxyamphetamine, also seem to be slightly more active – especially at low concentrations – after pre-treatment of the mice with pheniprazine No changes in the blocking potencies of noradrenaline or of 3 4-dihydroxynorephedrine and 3-hydroxynorephedrine were obtained after such pre treatment

Discussion

Several of the results obtained by Dengler and coworkers (DENGLER, SPIEGEL & TITUS 1961a & b DENGLER *et al* 1962) were confirmed They

Compounds with a side-chain of the propanolamine type seem to be more potent in blocking the uptake of tritiated noradrenaline by brain cortex tissues than are those with side-chains of other types. An exception was norephedrine, which was less active than amphetamine. Except for deoxyephedrine, one hydroxyl group on the β-carbon increased the activity. The potencies decreased when the substituent on the nitrogen was larger than a methyl group. Dihydroxyphenyl derivatives were more potent than m-hydroxyl analogues, which were in turn more active than the p-hydroxyl derivatives. Least active of all were amines without any hydroxyl in the benzene nucleus. Thus, there seems to be a distinct relationship between the adrenergic α-receptor-stimulating actions and the blocking effects of the amines studied by means of the uptake of tritiated noradrenaline.

The uptake mechanism studied does not seem to differentiate between the stereoisomeric forms of noradrenaline, since no difference was found between the inhibitory effect of the L- and D-isomers of noradrenaline on the uptake of tritiated noradrenaline. This finding is in agreement with that of KOPIN & BRIDGERS (1963), who found that both L- and D-noradrenaline-^{3}H were taken up and bound by rat heart and spleen at approximately the same rate.

DENGLER, SPIEGEL & TITUS (1961 b) showed that several inhibitors of adrenergic α-receptor responses, *e.g.* dibenzyline phentolamine and chlorpromazine, also block the uptake of tritiated noradrenaline by cerebral cortex tissues *in vitro*. These findings, together with those for the investigation reported here, may support the hypothesis that the α-receptor response depends on a mechanism similar to that determining the uptake of noradrenaline. The possibility that the α-receptors constitute the uptake mechanism studied is excluded, since the D-isomers of several sympathomimetic amine had a strong inhibitory effect on the uptake reaction, whereas the α-receptor-stimulating action is weak. Another possible explanation of the findings would be that an uptake of the sympathomimetic amine had to precede the reaction with α-receptors. But little is known about the cellular locations of the adrenergic receptors. The findings of SAVOREN & SUTHERLAND (1963) indicate that at least some adrenergic β-receptors may be membrane-bound. An intracellular location of α-receptors is not unlikely. In that event an uptake reaction of the amine must precede the receptor response. However, the blocking effects of cocaine and reserpine on the uptake of tritiated noradrenaline *in vitro* (DENGLER, SPIEGEL & TITUS 1961 b) seem to contradict this explanation, since these compounds have no α-receptor inhibitory effect.

Finally, there remains the possibility that the reaction determining the binding of the sympathomimetic amines to the α-receptors may be closely

naline taken up by preincubation of tissues *in vitro* (unpublished observation) Tyramine is one of the amines with the steepest concentration-response curves in our experiments. Other amines with the same kind of response curve were amphetamine and phenylethylamine.

As shown from our results, there were no distinct limits between amines with steep and less steep concentration response curves. All transitional forms can be observed between the two extremes of adrenaline on one hand and phenylethylamine on the other Several of the amines may have a mixed type of action.

From the above discussion it may be suggested that the concentration-response curves relating to sympathomimetic amines with an "indirect action on the receptors may exhibit steep slopes, whereas the curves of the amines with a direct" action on the receptors (BURN & TAINTER 1931 TRENDELENBURG 1963) may be less steep

Such a concept is supported by the results obtained. Amines with a mixed type of action on the receptors, *e g* ephedrine and norsynephrine, give curves with slopes intermediate between those of direct" and "indirect amines Also among the direct" acting amines some variations in slopes may be observed *e g* for adrenaline compared with synephrine.

Only few exceptions from this rule were found. According to the review of TRENDELENBURG (1963) dopamine would act as a direct" amine, but in our experiments it behaved as an indirect" one. However HARRISON, LEVITT & UDENFRIEND (1963) recently demonstrated that dopamine may at least in part, act by release of noradrenaline this is more constant with our results. In our experiments the curve of DL phenylethanolamine had a slope similar to that of synephrine and may therefore be classified as a predominantly direct acting amine BURN & RAND (1958) found that the L form of this compound behaves as a direct acting amine whereas the D-isomer has a predominantly "indirect action.

Specific exceptions to the rule that direct acting amines had less steep concentration response curves were the amines predominantly acting on adrenergic β-receptors. Isoproterenol the most potent of such amines, undoubtedly has a direct action on these receptors, but its concentration-response curve had a relatively steep slope. It also had only a slight blocking effect on noradrenaline uptake probably reflecting its slight α receptor-stimulating action (BUTTERWORTH 1963) which may be of the indirect type. N Ethylnoradrenaline on the other hand, had a slope similar to that for adrenaline and was also a relatively powerful inhibitor of noradrenaline uptake. In addition to its β-receptor stimulating action this amine may therefore be held to act on α receptors in a direct" way N Butylnoradrenaline, like the other β-receptor stimulating amines tested had only slight blocking action.

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similar to the binding mechanism between the amines and the hypothetical carrier structure in the uptake reaction. BELLEAU (1958) suggested that the α receptor reaction is mediated by binding of the amine to some acid structure of the receptors, e.g. carboxylic or phosphate ions. The binding of the amine to the carrier structure may be of a similar type.

Summary

The uptake of tritiated noradrenaline by cerebral cortex slices from mice was studied. Judging from kinetic experiments, this uptake resembles an enzymatic reaction.

The blocking actions of several sympathomimetic amines on noradrenaline uptake were investigated and showed good correlation with their α receptor stimulating effects. β-Receptor stimulating amines had only a poor inhibitory effect on noradrenaline uptake. Concentration-effect curves show that the "direct" receptor stimulating amines exhibit less steep curves than those with an "indirect" mode of action. The corresponding curves for amines with a mixed type of receptor action had slopes lying between these extremes.

Acknowledgements

For generous supplies of N-ethylnoradrenaline, N-butylnoradrenaline, norsynephrine and D- and L-norphenylephrine we thank Dr. F. P. Luduena and Dr. L. S. Harris, Sterling Winthrop Research Institute, Rensselaer, N.Y., U.S.A. We also thank Dr. H. Corrodi, AB Hässle, Gothenburg, Sweden, for the gift of 3-hydroxyamphetamine and D-3-hydroxynorephedrine.

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Table I

Content of amino acid in the food pellets (g/kg).

Aspartic acid	10.8
Threonine	5.2
Serine	5.1
Proline	7.7
Glutamic acid	37.7
Glycine	7.1
Alanine	7.8
Valine	7.5
Cystine	8.7
Methionine	4
Isoleucine	5.5
Leucine	10.7
Tyrosine	14.1
Phenylalanine	5.5
Lysine	5.5
Histidine	2.1
Arginine	5.4
H N	2.3

Feed

The animals were maintained on nutritionally sufficient pellets (FOA) ad lib. The amino acid contents of these pellets are recorded in table 1.

Experimental procedure

The mice were divided into groups of five. Some groups were subjected to 24 hours starvation immediately before the experiment, thus presumably producing a slight decrease in liver cystine, cysteine and methionine[1]). For exposure to chloroform, all animals were placed in a sealed air chamber of 3 litre capacity. A 0.20 ml constant mixture of labelled and unlabelled $CHCl_3$ was injected into a piece of cotton placed in a small net cage under the lid and allowed to evaporate for 20 minutes. Starved and fed mice were anaesthetized simultaneously to eliminate extraneous radioactive effects.

Subsequent to anaesthetization, each group was beheaded after interval t (min.) where $t = 0$, $t = 10$, or $t = e^n$ and $n = 3, 4, 5, 6, 7, 8$ or 9. The organ or organs to be studied were immediately dissected out, rinsed with water, dried, weighed in a sealed bottle and covered with 5 ml toluene. The tissue was then homogenized and the radioactivity of the homogenate determined. Temperature and external environment were held as constant as possible throughout the experiment.

Radioactivity Measurements

The time per 1000 β-particle emissions from each tissue homogenate was measured by means of a Geiger Muller tube. Backgrounds, amounting to approximately 30 cpm, were determined before and after each daily measurement. Since a constant isotope dilution was employed throughout, the *relative activities of the various* homogenates could be determined without the use of standards. The total activity of the labelled $CHCl_3$ was approximately 20 μC.

[1]) When deprived of food for 48 hours, rats manifest a 20 per cent decrease in total liver protein store (Addis *et al.* 1936).

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From the Department of Clinical Biochemistry (Dr Bertil Åberg) and the Department of Physiology Royal Veterinary College (professor Yngve Zotterman) Stockholm, Sweden.

Distribution of Chloroform-^{36}Cl in Mice

By

Göran Hellekant

(Received May 8 1964)

Although chloroform has been largely replaced by other anaesthetics, it has not lost its value altogether In an extensive clinical comparison, K. L. SIEBECKER *et al* (1960) found little difference between the effects of chloroform ($CHCl_3$) and fluothane and concluded that $CHCL_3$ remains useful for anaesthesia in many circumstances (*e g* when a rapidly acting anaesthetic is required for a brief period)

One of the main objections to the use of chloroform has been its apparent toxicity to the liver (GOODMAN & GILMAN 1955) This toxicity is greatly enhanced in animals depleted of the sulphur-containing amino acids (MILLER *et al* 1940 VICTOR 1952 NAKAMURA *et al* 1958). Since it is reasonable to suppose that the injurious effects of $CHCl_3$ are due to its halogen component, the radioactive isotope, ^{36}Cl can be readily employed to study the changes in liver concentration over time, compared with the changes in other tissue ^{36}Cl concentration and with the changes in ^{36}Cl liver concentrations in animals depleted of the sulphur-containing amino acids after brief chloroform anaesthesia. A knowledge of these functions should clarify the toxic effects of $CHCl_3$ on the organism.

Method and Materials

Isotope

$CH^{36}Cl_3$, supplied by the Radiochemical Center Amersham, England, was used. The half life of ^{36}Cl is $4\,4 \times 10^5$ years, and the energy of the β-emission is 0.714 MeV

Animals.

Eighty-two mice of both sexes (av wt 23 327 g range 16.529–31 720 g) were used.

Table 2

Radioactivity in organs at various times after inhalation of $CH^{36}Cl$.

Number of Mice	Time after Exposure	Sex M/F	Brain Counts/g/min M. S.d.	Liver Counts/g/min M. S.d.	Lung Counts/g/min M. S.d.	Kidney Counts/g/min M. S.d.
5	0 min.	5/0	19.1 ± 2.6	28.7 ± 8.8	19.3 ± 17.8	19.4 ± 4.8
4	10 min.	1/3	4.9 ± 4.9	14.9 ± 8.4	3.2 ± 1.7	7.6 ± 2.7
4	20 min.	2/2	30.9 ± 20.6	20.0 ± 7.6	77.4 ± 40.0	72.6 ± 61.0
5	54 min.	2/3	66.1 ± 97.0	37.1 ± 33.1	65.2 ± 25.9	36.7 ± 33.5
5	2 hrs. 28 min.	2/3	22.0 ± 31.6	16.0 ± 11.0	75.0 ± 41.8	27.8 ± 23.8
3	6 hrs. 45 min.	1/2	27.1 ± 14.1	8.9 ± 5.9	32.3 ± 40.5	28.6 ± 13.2
5	18 hrs. 20 min.	2/3	8.4 ± 5.2	8.9 ± 6.2	24.7 ± 21.0	10.3 ± 11.5

A comparison between the liver chlorine concentrations in fed and starved mice (fig. 2) showed significant differences ($0.01 < p < 0.02$, Student's t-test, two-tailed) immediately after the chloroform exposure. Ten minutes later the difference was not statistically significant ($p > 0.1$) and 20 minutes after the exposure there were no differences at all between the groups.

Discussion

That ^{36}Cl-induced activity had disappeared from the liver approximately 2 days after exposure to labelled chloroform is in good agreement with the reports of other investigators. SOUCEK (1962) has stated that chloroform administered anaesthetically is entirely dissipated from the organism within a few days. Further extrapolation of the curve of CHENOWETH *et al* (1962) for respiratory elimination supports the predic

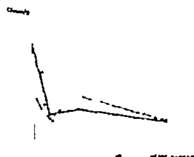


Fig. Decrease in ^{36}Cl activity of livers of fed and fasted animals. ● ● fed group fasted group. The numbers in the figure indicate number of animals corresponding groups.

Statistical Methods

The results were analysed by appropriate statistical methods. Total counting errors due to background variation were calculated from the formula s.d. of sample counting rate

$$= \pm \sqrt{\frac{C_s}{T_s^2} + \frac{C_B}{T_B^2}}$$

where C_s = total counts for sample in time T and C_B = total counts for background in time T_B.

In this investigation the standard deviation of sample counting-rate, when not stated, varied between 1 and 8 per cent.

Results

After brief exposure to chloroform, the radioactivity of ^{36}Cl per gram liver tissue decreased approximately linearly with time (fig. 1) in a semi-logarithmic plot When one considers background and individual variations, it is apparent that after 50 hours ^{36}Cl had entirely disappeared from the liver of many animals. Activity measured after 5 days + 16 hours can be wholly attributed to background variation which was ±1.2 cpm/g at this point.

Changes in brain liver lung and kidney ^{36}Cl activity apparently increased noticeably over immediate post-exposure values *before decreasing* toward zero as would be expected (table 2) However individual variations were too great for this result to be statistically significant. Of the organs studied, liver ^{36}Cl concentrations were the most constant, those of the lung most variable with time and among individuals at one time.

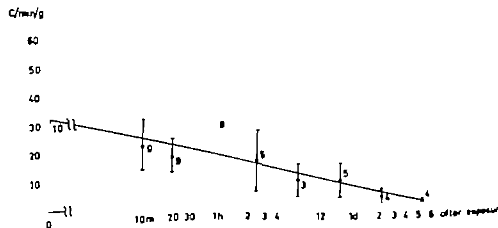


Fig. 1 Decrease of ^{36}Cl in the liver The numbers in the figure indicate number of animals in corresponding groups. Standard deviations in brackets. The time axis (ordinate) is logarithmic (see Methods).

Table 2

Radioactivity in organs at various times after inhalation of $CH^{14}Cl_3$

Number of Mice	Time after Exposure	Sex M/F	*Brain* Counts/g/min M S.d.	*Liver* Counts/g/min M.v. S.d.	*Lung* Counts/g/min M.v S.d.	Kidney Counts/g/min M. S.d.
5	0 min.	5/0	19.1 ± 2.6	28.7 ± 8.8	19.3 ± 17.8	19.4 ± 4.8
4	10 min.	1/3	4.9 ± 4.9	14.9 ± 8.4	3.2 ± 1.7	7.6 ± 2.7
4	20 min.	2/2	30.9 ± 20.6	20.0 ± 7.6	77.4 ± 40.0	72.6 ± 61.0
5	54 min.	2/3	66.1 ± 97.0	37.1 ± 33.1	65.2 ± 25.9	36.7 ± 33.5
5	2 hrs. 28 min.	2/3	22.0 ± 31.6	16.0 ± 11.0	75.0 ± 41.8	27.8 ± 23.8
3	6 hrs. 45 min.	1/2	27.1 ± 14.1	8.9 ± 5.9	32.3 ± 40.5	28.6 ± 13.2
5	18 hrs. 20 min.	2/3	8.4 ± 5.2	8.9 ± 6.2	24.7 ± 21.0	10.3 ± 11.5

A comparison between the liver chlorine concentrations in fed and starved mice (fig. 2) showed significant differences ($0.01 < p < 0.02$, Student's t test, two-tailed) immediately after the chloroform exposure. Ten minutes later the difference was not statistically significant ($p > 0.1$) and 20 minutes after the exposure there were no differences at all between the groups.

Discussion

That ^{14}C-induced activity had disappeared from the liver approximately 2 days after exposure to labelled chloroform is in good agreement with the reports of other investigators. SOUCEK (1962) has stated that chloroform administered anaesthetically is entirely dissipated from the organism within a few days. Further extrapolation of the curve of CHENOWETH *et al* (1962) for respiratory elimination supports the predic-



Fig. Decrease in ^{14}C activity of livers of fed and fasted animals ● ● fed group — fasted group. The numbers in the figure indicate number of animals in corresponding groups.

tion that exhalation of $CHCl_3$ will terminate between 1 and 2 days after exposure

The increase in ^{36}Cl activity of lungs liver brain and kidney after about 30 minutes is difficult to interpret. That it was produced by an artifact is unlikely although individual variation was large the effect was also large and was repeatedly observed over the course of the experiment. Nor is this result inconsistent with previous reports, for most investigators have failed to make concentration determinations within the first hour after exposure to chloroform $CHCl_3$ content does not decrease for several hours in other tissues, such as subcutaneous fat (CHENOWETH 1967). At any given time after chloroform inhalation, tissue concentrations of $CHCl_3$ and its chloride derivatives are undoubtedly a function of two mechanisms those contributing to their build up such as anaesthetic concentration duration of exposure and rate of transport and those contributing to its elimination Most of the inhaled $CHCl_3$ is exhausted through respiration (SOUCEK 1962) but to a lesser extent it is metabolized in the body or excreted through other systems or both BUTLER (1961) has observed the presence of $CHCl_3$ in expired air from dogs exposed to CCl_4 and has reported the formation of CH Cl tissue treated with chloroform and cysteine *in vitro* Besides such general effects variation in the concentration time course from tissue to tissue due to specific properties of the organs involved would be expected. Thus the large standard deviation observed in lung ^{36}Cl content of animals from the same group might be partly attributable to differences in the amount of blood pouring into the lung at death Similarly the amount of activity before death hence the extent of ^{36}Cl accumulation in the lung physiological dead space" would be expected to vary both between groups and among individuals. It is reasonable to assume that, if all such factors were understood, the observed increase in tissue ^{36}Cl concentration could be more readily interpreted.

The time course of ^{36}Cl induced activity in the liver is a case in point for here additional knowledge of elimination mechanisms is available The specific role of cysteine and methionine in preventing liver injury after administration of $CHCl_3$ (MILLER *et al* 1942 GLYNN *et al* 1945) suggests that the sulphur-containing amino acids are of critical importance to the metabolism and elimination or at least to the inactivation, of $CHCl_3$ in the liver In this investigation it was assumed that a 24-hour food deficit would significantly diminish the liver sulphur amino acid store this assumption was based on observations of analogous phenomena in rats (ADDIS *et al* 1936 LEAF 1947) along with the consideration that mice manifest higher metabolic rates hence equivalent amino acid deficits would occur in these within a shorter deprivation period GOLD-

SCHMIDT *et al.* 1939 found after one hour of exposure to chloroform, more liver injury in rats starved for 24 hours than in fed rats.

This investigation shows that mice deprived at food exhibit significantly higher liver ^{36}Cl contents than fed mice for the first ten minutes after exposure to $CHCl_3$. If the assumption is correct, these results give rise to the hypothesis that during chloroform inhalation the concentration of ^{36}Cl in the liver varies directly with total $CHCl_3$ absorption in the lung, equal in fed and starved groups, and negatively with the concentration of SH-groups available for combining in the liver. Since starved animals have fewer available SH-groups than ones that have eaten, their liver $CHCl_3$ content is higher immediately after anaesthetization. Then when chloroform intake has ceased, the amount of ^{36}Cl found, in the liver depends solely upon the SH reaction. At just this moment the rate of the reaction becomes primarily a function of chloroform concentration and accordingly will be more rapid in starved than in fed animals. Eventually the liver chloroform in both is reduced to such a level that the SH groups again assume dominance in determining the breakdown rate. But since liver stores of sulphur amino acids have been exhausted, the reaction can occur only as fast as they can be brought from other parts of the body and will be the same for both groups.

Regardless of the mechanism involved, it appears clear from those results that prolonged food deprivation before chloroform anaesthetization should be avoided if liver injury is to be prevented.

Summary

Mice were exposed to ^{36}Cl-labelled chloroform for 20 minutes with the results that

a) liver ^{36}Cl content disappeared after approximately two days.

b) An unexplained increase in ^{36}Cl concentration was observed in lung, kidney, brain and to some extent liver after approximately 30 minutes.

c) Mice deprived of food for twenty four hours showed greater liver ^{36}Cl contents than fed animals for at least 10 minutes after anaesthetization. It is concluded that the food deprivation period before chloroform exposure should be relatively short so as to avoid the liver damage often associated with this anaesthetic.

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Selective Uptake of Some Catecholamines by the Isolated Heart and its Inhibition by Cocaine and Phenoxybenzamine

By

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and Henry Persson*)

(Received May 16, 1964)

Reserpine depletes the noradrenaline in sympathetically innervated tissues (Bertler, Carlsson & Rosengren 1956 Burn & Rand 1957 Muscholl & Vogt 1958). After reserpine treatment the sympathetic nerves do not release noradrenaline on electrical stimulation or after carbacholine injection (Bertler, Carlsson & Rosengren 1956 Carlsson *et al* 1957 Bertler *et al* 1958 Muscholl & Vogt 1958) Further the sympathomimetic effect of tyramine is abolished by reserpine (Carlsson *et al* 1957 Burn & Rand 1958 Trendelenburg 1961). In reserpine treated animals an infusion of noradrenaline or one of its precursors or analogues may temporarily restore the response to electrical stimulation of sympathetic nerves and to tyramine (Burn & Rand 1958 1960 Rosell & Sedvall 1961 Gillis & Nash 1961 Moore & Moran 1962 Mirkin & v Euler 1963). The same results have also been obtained by experiments on isolated organs (Bejrablaya, Burn & Walker 1958 Gillespie & Mackenna 1960 Crout Muskus & Trendelenburg 1962).

The starting point for the present investigation was the finding that isoprenaline, in contrast to noradrenaline and adrenaline, could not restore the tyramine effect in isolated hearts from reserpine treated rabbits. It was further shown that cocaine and phenoxybenzamine potentiated the responses to noradrenaline and adrenaline, but not to isoprenaline in hearts from normal rabbits. These findings prompted us to investigate whether adrenaline and isoprenaline could accumulate in isolated hearts from normal rabbits and whether cocaine or phenoxybenzamine affected this uptake.

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Material and Methods

Male and female rabbits weighing 2–3 kg were stunned by a blow on the neck and bled from the carotid arteries. The heart was removed as rapidly as possible.

Isolated heart The heart was placed in a perfusion apparatus similar to that described by KARP RINZLER & TRAVELL (1960). The apparatus is shown diagrammatically in figure 1 The coronary system was perfused, as described by Langendorff, with Krebs solution aerated by carbogen (94 / O_2 and 6 / CO_2) it had attained a temperature of 37°C in a heating coil before entering the heart. The perfusion was performed by means of a sigmamotor pump (zero-Max model T 8). The perfusion pressure was recorded on a smoked drum by a mercury manometer and was about 20 mm Hg during the experiment. The flow rate was 9–12 ml/min. and was kept constant by the pump throughout The suspended heart was surrounded by a water jacketed bath (37°C) which protected it from cooling. On the smoked drum the contractions of the heart were recorded by means of a pulley and lever system and the heart rate by an ordinate writer (constructed as described by A. Fleisch and manufactured by B. Braun Melsungen) The latter was operated by a photoelectrical relay whose circuit diagram is given in fig. 2. A light beam is received from the bulb (A) and is focused on a photodiode (B). The lever (C) is attached to the heart. When the lever is in the down position the beam is interrupted, and no current passes through the transistor (D). When the lever is in the up position, the light beam will pass uninterrupted to the photodiodo (B) the current increases in the transistor (D) and the relay starts to function. This breaks the contact between E and F and the ordinate writer returns to the zero level The diode (G) short circuits induction currents produced in the relay coil and thereby protects the transistor from overloading.

The sympathomimetic amines were added to the perfusing fluid just before the entry to the heart by injection or infusion through a rubber sleeve. Cocaine and phenoxybenzamine were dissolved in the perfusion fluid. After reserpine treatment and in the biochemical experiments the catecholamines, *i e* noradrenaline, adrenaline and isoprenaline, were given by means of a perfusion pump (Unita II B. Braun). Otherwise the sympathomimetic amines were injected rapidly

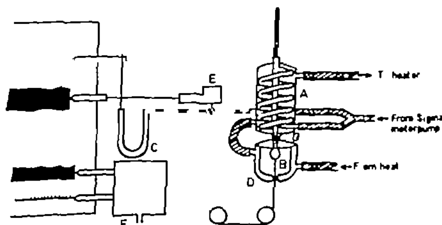


Fig. 1 Diagram of perfusion of the solated heart by Langendorff procedure
A. Heating coil
B. Heart
C. Mercury manometer
D Water jacketed bath
E. Photoelectric el y
F O dinate writer
G R bber leeve

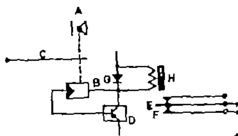


Fig. 2. Photoelectrical relay
A Bulb (12 V) C. Lever connected to the heart
B. Photodiode, OAP 12 (Philips) D Transistor OC 76 (Philips)
E and F Make and break in the relay (H), TBV 65417–93 (Siemens).

Papillary muscle The papillary muscles from the left ventricle were removed from the freshly isolated heart. One papillary muscle was suspended in a water-jacketed organ bath with Krebs solution at 37°C. The solution was oxygenated by carbogen before its passage through a heating coil into the bath, which also received a constant supply of carbogen. The muscle was electrically driven (Tetronic set consisting of power supply type 160, waveform generator type 162, pulse generator type 161 indicator type 360). The inotropic responses were recorded by means of a force displacement transducer (Swema model SG4-1 no. 10036) and an Offner dynograph (type 504B ser 103 sensitivity 100 or 200 µV per cm). The muscle was stimulated by rectangular wave pulses of 5–10 msec duration and 5 V strength at a frequency of 1–2 impulses per second. Well-shielded platinum electrodes were used. The sympathomimetic amines were added to the organ bath (volume 50 ml) every 15 minutes and remained in contact with the muscle for three minutes before washing out. The stimulation began some minutes before adding the sympathomimetic amines and continued as long as they were in the bath. Cocaine and phenoxybenzamine were put into the bath one minute before the catecholamines.

Drug treatment Reserpine was given intraperitoneally to the rabbits on three consecutive days. On the first two days the rabbits received 1 mg/kg and on the day before the experiment 3 mg/kg. The compounds used in the course of the experiment were ( )-adrenaline L-(+)-bitartrate, (−)-noradrenaline L-(+)-bitartrate, (−)-isoprenaline L-(+)-bitartrate tyramine hydrochloride, cocaine hydrochloride, phenoxybenzamine hydrochloride. The doses refer to the salt, except for the catecholamines, which are calculated as the free base. Unless otherwise stated the compounds were administered in 0.9 sodium chloride solution.

Biochemical determinations After the end of infusion of the catecholamines the coronary system was washed out for five minutes by Krebs solution. Immediately afterwards the heart was cut into pieces and dried on filter paper. The tissue was homogenized in 0.4 N perchloric acid (3–5 ml per g tissue). To 10 ml of the acid extract 2 mg ascorbic acid and 20 mg tetracemin-disodium were added. The sample (pH 6.5) was purified on Dowex 50 column (X4, 4.2 × 40 mm in sodium form). Differential assay of noradrenaline and adrenaline was performed by the method of BERTLER, CARLSSON & ROSENGREN (1958). Isoprenaline was determined in the same way as adrenaline, but had to be luted with somewhat larger volume of N HCl (12 instead of 9 ml).

Results

In the heart preparations from reserpine treated rabbits it was found that tyramine in moderate doses was ineffective, as previously found (fig. 3 for references, see above) If tyramine was given during an infusion of noradrenaline or adrenaline the response to tyramine obtained was as by hearts from untreated animals (fig. 3) After the infusions of the catecholamines had stopped the tyramine effect could still be observed for some minutes but it gradually decreased. Isoprenaline failed completely to restore the effect of tyramine in hearts from reserpine-treated rabbits (fig. 3) The doses used of noradrenaline, adrenaline and isoprenaline gave about equal direct inotropic and chronotropic effects.

In hearts from normal rabbits also it was possible to demonstrate a qualitative difference between noradrenaline and adrenaline on the one hand and isoprenaline on the other Cocaine or phenoxybenzamine at a concentration of 1 µg/ml in the perfusion fluid potentiated the effect of noradrenaline and adrenaline, but not that of isoprenaline in the Langendorff preparation (fig. 4 and 5) Nor did higher concentrations (10 µg/ml) enhance the action of isoprenaline. The potentiation of adrenaline was always less marked than that of noradrenaline The noradrenaline effect

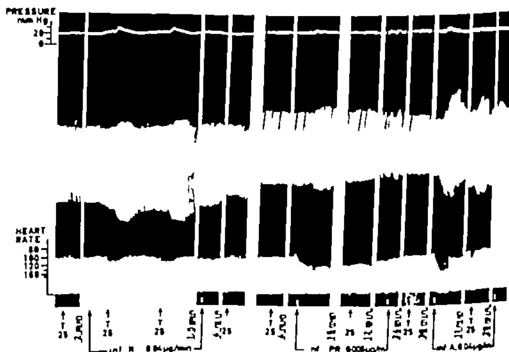


Fig. 3 The isolated rabbit heart, reserpine treated. The response to tyramine (T 25 µg) before and a few minutes after beginning inf sion of noradrenaline (NA, 0.04 µg min.), isoprenaline (IPR, 0.005 µg/min.) or adrenaline (A, 0.04 µg/min.). Note that the response to tyramine has disappeared already four minutes after the end of noradrenaline infusion. Upper tracing perfusion pressure. Middle tracing heart contractions (down systole). Lower tracing heart rate.

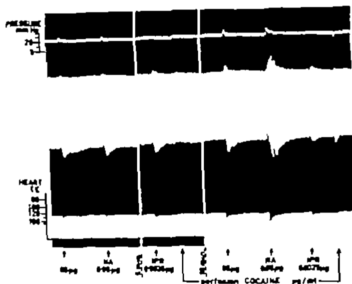


Fig. 4. Isolated normal rabbit heart. Response to adrenaline (A), noradrenaline (NA), and isoprenaline (IPR) before and during perfusion with solution containing cocaine 12 µg/min.). Tracings as in figure 3

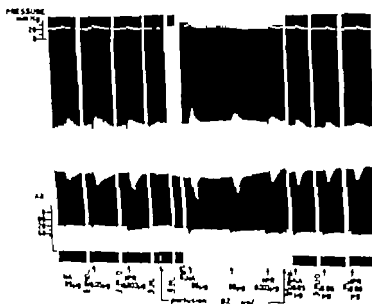


Fig. 5. Isolated normal rabbit heart. Response to noradrenaline (NA), adrenaline (A) and isoprenaline (IPR) before, during and after perfusion with solution containing phenoxybenzamine (PBZ, 12 µg/min.). Tracings as figure 3

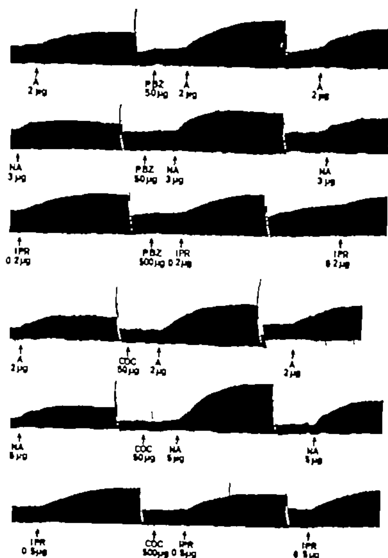


Fig. 6 Rabbit papillary muscle preparation. The response to adrenaline (A) noradrenaline (NA) and isoprenaline (IPR) before adding, during the presence of and after washing out phenoxybenzamine (PBZ, upper three tracings) or cocaine (COC lower three tracings).

was maximally enhanced to about four times the normal whereas that of adrenaline was only to about twice The potentiated responses to noradrenaline and adrenaline were readily eliminated by stopping the cocaine supply The effect of phenoxybenzamine did not seem to be equally reversible since the actions of noradrenaline and adrenaline, although diminishing, were enhanced for several hours or as long as we observed them after the end of its supply

In the papillary muscle preparations from rabbits it was found also that both cocaine and phenoxybenzamine at a concentration of 1 µg/ml in the bath enhanced the effect of noradrenaline and adrenaline but not that of isoprenaline (fig. 6) Even higher concentrations of these compounds did

Table 1

Levels of catecholamines in μg/g in the isolated rabbit heart perfused for one hour with Krebs solution containing the compounds listed to the left. Figures in parentheses represent the total amount given (see treatment column) and the percentage of administered catecholamine taken up by the heart (see adrenaline or isoprenaline column).

Treatment	Noradrenaline	Adrenaline Isoprenaline
Untreated	2.17	0.03
	2.05	0.04
	2.08	0.03
	2.70	−0.01
(−)-Isoprenaline (4 μg)	2.49	0.01
	2.13	0.02
	1.91	0.02
(−)-Adrenaline (6 μg)	2.21	0.55 (28%)
	2.37	0.39 (25%)
	2.37	0.24 (12%)
	1.38	0.31 (24%)
Cocaine HCl (2.7–3.6 mg) + +(−)-Adrenaline (6 μg)	1.35	0.01
	1.43	0.04
Phenoxybenzamine HCl (2.7–3.6 mg) + +(−)-Adrenaline (6 μg)	1.20	0.00
	0.93	0.05

Adrenaline and isoprenaline values of 0.05 μg/g and lower do not differ significantly from zero.

not potentiate the isoprenaline response. The effects of both cocaine and phenoxybenzamine did not seem to be irreversible, since they were reduced by repeated washing.

In studies in which the uptake of the catecholamines was investigated the hearts were perfused with the amines for one hour with or without the potentiating compounds. The results are shown in table 1. In the hearts from untreated animals from 12 to 28 per cent of the infused 6 μg adrenaline was found in the heart five minutes after the one-hour long administration. Isoprenaline was given in an amount of 4 μg during one hour. This dose was the largest that the heart could sustain and was between one twentieth and one tenth of the dose of adrenaline producing the same increase in rate and force of the heart contractions. The uptake of isoprenaline was insignificant, *i. e.* no larger than the amounts of adrenaline found in the untreated hearts by the method used. The uptake of adrenaline was apparently completely blocked by cocaine and phenoxybenzamine at a concentration of 5 μg/ml in the perfusion fluid.

Discussion

Injections of labelled or unlabelled adrenaline or noradrenaline cause an accumulation of these amines in sympathetically innervated tissues

(AXELROD WEIL MALHERBE & TOMCHICK 1959 MUSCHOLL 1960 WHITBY AXELROD & WEIL MALHERBE 1961 STRÖMBLAD & NICKERSON 1961 ANDÉN 1964) Also the perfused heart has been shown to take up noradrenaline (KOPIN HERTTING & GORDON 1962 IVERSEN 1963). The uptake of these amines probably occurs in sympathetic nerves, since it disappears after postganglionic denervation (HERTTING *et al.* 1961 STRÖMBLAD & NICKERSON 1961 ANDÉN CARLSSON & WALDECK 1963). The uptake of catecholamines by the sympathetic nerves has also been demonstrated directly by a specific histochemical fluorescence method for monoamines (HAMBERGER *et al* 1964) and by electron microscope autoradiography (WOLFE *et al* 1962)

It is well known that cocaine potentiates the effect of injections of adrenaline (FRÖHLICH & LOEWI 1910) or noradrenaline (FLECKENSTEIN & BASS 1953) Parallel with the enhanced responses to injected noradrenaline and to submaximal sympathetic stimulation the half life of injected noradrenaline in plasma is prolonged after cocaine (TRENDELENBURG 1959) MACMILLAN (1959) suggested that the potentiation by cocaine is due to a blockade of the uptake of noradrenaline and adrenaline in the tissue stores. Later it was experimentally found that cocaine in fact inhibited noradrenaline uptake by the heart and other tissues (WHITBY HERTTING & AXELROD 1960 MUSCHOLL 1961 IVERSEN 1963) Further phenoxybenzamine has been shown to potentiate the effect of noradrenaline in the heart (FURCHGOTT 1959 BENFEY & GREFF 1961 FURCHGOTT & KIRPEKAR 1963) and to inhibit its uptake (HERTTING, AXELROD & WHITBY 1961) As is well known phenoxybenzamine blocks the adrenergic α receptors whereas the response to noradrenaline in the heart is due to a stimulation of the β-receptors

It has been found that cocaine increases the output of noradrenaline to the venous blood or the perfusing fluid when the sympathetic nerves are stimulated at moderate frequences (NASMYTH & ANDREWS 1959 HUKOVIC & MUSCHOLL 1961 KIRPEKAR & CERVONI 1963) However the output is increased much more after phenoxybenzamine (BROWN & GILLESPIE 1957 KIRPEKAR & CERVONI 1963) The larger output of noradrenaline after phenoxybenzamine may be explained if the adrenergic α receptors are involved in the removal of the transmitter (BROWN & GILLESPIE 1957) However the blockade of the vasoconstrictor action of noradrenaline by phenoxybenzamine may increase the elimination of noradrenaline by diffusion into the blood vessels (ROSELL, KOPIN & AXELROD 1963) Recent experiments suggest that the last mentioned mechanism is of importance (CARLSSON FOLKOW & HÄGGENDAL, unpublished observations)

The results of the present investigation show that the potentiation of

noradrenaline and adrenaline effects by cocaine and phenoxybenzamine is due to a fairly specific mechanism, inasmuch as there is no enhancement of the action of isoprenaline, closely similar in structure. Since it is probable that all three catecholamines stimulate the same receptors in the heart, it is not likely that the selective potentiation of noradrenaline and adrenaline is due to action of cocaine or phenoxybenzamine at the receptor sites, as has been suggested (MAXWELL *et al* 1959) While this work was in progress, the same observations were reported by STAFFORD (1963). She found this phenomenon also after administration of guanethidine. The inability of isoprenaline to restore the action of tyramine in isolated reserpine-treated hearts has also been described earlier (KUSCHINSKY *et al.* 1960). The demonstrated lack of uptake of isoprenaline, in contrast to the marked one of adrenaline, seems to offer a reasonable explanation of these findings. The undetectable uptake of isoprenaline cannot be due to the fact that the dose of isoprenaline used was somewhat smaller than that of adrenaline, since it has been found that the amine uptake calculated as a percentage of the amount perfused diminishes when the quantity of the amine administered increases (IVERSEN 1963) Also, with the histochemical method for detection of monoamines it has been observed that the sympathetic nerves fail to take up isoprenaline, whereas there is in the nerves a marked accumulation of adrenaline given in the same dose (SACHS, unpublished observation) We have found that, after administration of a large dose of (±)-isoprenaline (40 mg/kg i p to rats), there is an uptake of the administered amine by the heart and a depletion of the noradrenaline to about half the normal in two hours. Probably the tissues are thereby exposed to much more isoprenaline than they were in the *in vitro* experiments reported here.

It has been found that restoration of the response to tyramine in reserpine-treated animals does not depend on replenishment of the noradrenaline stores (MUSCHOLL 1960). This fact, it has been suggested, indicates that the action of tyramine is at least partly due to the presence of extraneuronal noradrenaline (MUSCHOLL 1960 KUSCHINSKY *et al.* 1960 NASMYTH 1962 SMITH 1963) However the inability to restore the tyramine response by isoprenaline after reserpine treatment, or by noradrenaline after postganglionic sympathectomy (BURN & RAND 1960), suggests that the primary site of action of tyramine is the adrenergic nerve terminals. The response to tyramine, despite an unmeasurable or extremely small (CROUT MUSKUS & TRENDELENBURG 1962) noradrenaline uptake, is compatible with the view that tyramine liberates noradrenaline from an extremely small "available" store (TRENDELENBURG 1961 CROUT MUSKUS & TRENDELENBURG 1962).

Cocaine does not prevent the active incorporation of monoamines into

isolated adrenomedullary granules (CARLSSON, HILLARP & WALDECK 1963). Phenoxybenzamine added *in vitro* is a fairly potent inhibitor of this uptake mechanism (CARLSSON, HILLARP & WALDECK 1963), but seems to be ineffective *in vivo* (LUNDBORG 1963). Cocaine and phenoxybenzamine have been stated to block "transfer sites" situated in or very close to the cell membrane of the adrenergic nerve terminals (FURCHGOTT & KIRPEKAR 1963, FURCHGOTT *et al.* 1963) and tyramine has been supposed to act there too (FURCHGOTT & KIRPEKAR 1963). The tyramine effect is abolished by treatment with cocaine (TAINTER & CHANG 1927), as well as with phenoxybenzamine, even in the heart (BENFEY & GREFF 1961). The "transfer sites" have been thought the most important "sites of loss" of noradrenaline in the synaptic gap (TRENDELENBURG 1963). LINDMAR & MUSCHOLL (1963) have found that about 26% of 500 ng noradrenaline perfusing the rat heart for 10 minutes is taken up in the tissue and that the accumulated noradrenaline amounted to about 75% of that not appearing in the perfusate. Noradrenaline seems to be taken up in the tissues to a somewhat larger extent than adrenaline (WHITBY, AXELROD & WEIL-MALHERBE 1961). Our findings that the normal transmitter, noradrenaline, appears to be taken up better than adrenaline, and that the uptake of isoprenaline is negligible, support the view that uptake by the nerve terminals is one of the most important routes of inactivation of the adrenergic transmitter.

Summary

About 25% of perfused adrenaline accumulated in the isolated rabbit heart, whereas there was no detectable uptake of isoprenaline. The lack of uptake of isoprenaline probably explains why this substance, in contrast to adrenaline and noradrenaline, failed to restore the sympathomimetic effect of tyramine in isolated hearts from reserpine treated rabbits and was not potentiated by cocaine and phenoxybenzamine in the isolated rabbit heart or in the rabbit papillary muscle preparation. The uptake of adrenaline by the isolated rabbit heart was completely blocked by cocaine and phenoxybenzamine. These findings are consistent with the hypotheses that 1) uptake by the nerve terminals is an important route of inactivation of the adrenergic transmitter, 2) cocaine and phenoxybenzamine potentiate the effect of noradrenaline and adrenaline by blocking this uptake, 3) tyramine acts by releasing noradrenaline from the postganglionic sympathetic nerve terminals.

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On the Mechanism of Noradrenaline Depletion by α Methyl Metatyrosine and Metaraminol

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The tissues can be depleted of the monoamines 5-hydroxytryptamine (5HT), dopamine (DA) and noradrenaline (NA) by drugs that seem to act differently both biochemically and functionally. Two groups of drugs have been extensively studied, namely a) the *Rauwolfia* alkaloids and certain benzoquinolizines, and b) the DOPA analogues, α methyl DOPA and α methyl metatyrosine (αMMT). The drug of the first group most employed is reserpine. This agent depresses the levels of all the three monoamines to about the same extent and for about the same time (for references, see SHORE 1962 CARLSSON 1964b). Further it causes sedation, extrapyramidal disorders and reduced sympathetic tone. Though α methyl DOPA can elicit similar but much weaker signs, αMMT fails almost completely to produce them. Also the effect of αMMT to lower the monoamine concentrations differs from that of reserpine. There is a transient drop of 5HT and DA in the brain after administration of αMMT and the NA levels in the central nervous system and in the sympathetically innervated organs are markedly reduced for several days (HESS *et al* 1961 PORTER *et al* 1961 SOURKES *et al* 1961 CARLSSON & LINDQVIST 1962 COSTA *et al* 1962a). The differences between the actions of reserpine and of αMMT have prompted investigation into the mode of action of αMMT to reduce the monoamine levels. CARLSSON & LINDQVIST (1962) found by paper chromatography that αMMT is decarboxylated to α methylmetatyramine, which is subsequently β-hydroxylated to metaraminol (α methyl β-hydroxymetatyramine) at the NA storage sites. The amounts observed of α methylmetatyramines roughly corresponded to the amounts of the

Some of the results of this investigation were reported at the Second International Pharmacological Meeting, Prague, August 21 1963

mixing naturally occurring amine analogues, *i. e.* the drop in amine levels appeared to be due to displacement by the decarboxylation products. However UDENFRIEND & ZALTZMAN-NIRENBERG (1962) as well as GESSA *et al.* (1962b) were unable to detect α-methylmetatyramines in quantities that balanced the long-lasting loss of NA. The purpose of our study has therefore been to investigate whether or not the α-methylmetatyramines formed from αMMT can be found in amounts stoichiometrically equal to those of the lost amines. Since UDENFRIEND & ZALTZMAN-NIRENBERG (1962) as well as GESSA *et al.* (1962a) have found that injections of metaraminol also can produce depletion of heart NA, a few experiments were undertaken with this drug.

Material and Methods

Adult rats, rabbits and guinea pigs were used. The rats were killed by beheading them, the rabbits by the intravenous injection of air and the guinea pigs by a blow on the back of the neck. The brains and hearts from 3 rats, the brains (divided into the cerebral hemispheres, the corpora striata, the brain stem and the cerebellum) from 2 rabbits and the hearts from 2 guinea pigs were removed as fast as possible. The tissues were rapidly extracted with 4–6 ml ice-cooled 0.4 N perchloric acid per g. Careful metal homogenization was performed by an "Ultra-Turrax". The precipitate was spun down in a refrigerated centrifuge (10,000 × g, 10 min., 0°C). After filtration of the supernatant, the residue was re-extracted with about 4 ml ice-cooled 0.4 N perchloric acid per g. The two extracts were pooled after measuring their volumes.

To 10 ml of the total extract 0.1 ml 2% ascorbic acid and 0.2 ml 10% EDTA (tetracemin-dinatrium) were added. Then the sample was treated as described by CARLSSON & LINDQVIST (1962). The elution was performed in 3 steps: 4 ml N-HCl + 12 ml N-HCl + 17 ml 2N-HCl. For the NA assay 1 ml of the first and 3 ml of the second eluate were pooled. The rest of the second eluate was used for the determination of metaraminol. The third eluate contained the α-methylmetatyramine. The DA estimations were performed on measured volumes of the second and third eluates.

The eluates containing the α-methylmetatyramines were evaporated to dryness under reduced pressure at 37°C (external) in a rotating round bottom flask. The residue was taken up in 2 ml redistilled water. The solution was transferred to a 7.5 ml glass-stoppered centrifuge tube. The rest of the determination was performed essentially by the method of UDENFRIEND & ZALTZMAN-NIRENBERG (1962) and GESSA *et al.* (1962a). There were then added 0.5 ml 0.5 M sodium borate buffer (pH 9.3) and 0.1 ml Gibbs reagent (0.05% N,2,6-trichloro-p-quinoneimine in 99% ethanol, prepared freshly before use). After standing for 10 minutes the colour was extracted with 1 ml n-butanol. (The butanol had been purified by successive washings with equal volumes of N-NaOH, N-HCl and redistilled water to remove the acid.) After centrifugation the absorbance at 655 mμ of the chromophore in the butanol phase was determined in a Beckman DU spectrophotometer. The readings were always completed within 60 minutes of the addition of the Gibbs reagent.

Standards of metaraminol and α-methylmetatyramine as well as a reagent blank were always treated in the same way as the eluates. Under these conditions 1 μg of metaraminol and of α-methylmetatyramine had absorbances of about 0.160 and

0.225 respectively The samples obtained from tissues of untreated animals had low absorbances. The corresponding amounts of α-methylmetatyramines were calculated and are shown in brackets in the tables. The values of these tissue blanks were always subtracted from those of the samples. The recoveries of 1–2 μg of amines added to the tissues before homogenization are also given in the tables. All values were corrected accordingly In the rabbit experiments it was assumed that the recoveries were equal from the cerebellum and the other examined tissues.

The NA was determined as described by BERTLER, CARLSSON & ROSENGREN (1958) and the DA by the method of CARLSSON & WALDECK (1958) as modified by CARLSSON & LINDQVIST (1962) The 5HT was assayed on measured portions of the tissue extract as described by BERTLER (1961) and CARLSSON & LINDQVIST (1962).

Results

1 *Amine levels in the rat brain after αMMT*

The time course of the changes in brain amine levels was followed in rats after intraperitoneal injection of αMMT at a dose of 400 mg/kg (DL form) The NA concentration dropped by some 80 / within 6 hours and remained low then rising slowly for several days (table 1) Metaraminol occurred in the rat brain as long as the NA level was reduced, and the amount of missing NA roughly corresponded to the amount of metaraminol recovered However except at 6 hours the amount of metaraminol during the first day seemed to be somewhat smaller than

Table 1

Levels of noradrenaline (NA) metaraminol (MA) 5-hydroxytryptamine (5HT), dopamine (DA) and α methylmetatyramine (αMMTA) in the rat brain at various times after 400 mg/kg intraperitoneal DL-α methyl metatyrosine. The normal values are a erages _s e.m. from three experiments on nine rats. All values are corrected for reco ery nd tissue blank (n bracket)

Time after injection	NA in nmol/g	NA deficit in nmol/g	MA in nmol/g	MA in of the NA deficit	5HT in nmol/g	DA in nmol/g	5HT + DA deficit in nmol/g	αMMTA nmol/g
0	2.6 ± 0 11 Recovery = 97 /		(0 16) Reco ery = 78		2.6 0 02 Recovery 78	7 0 0.48 Recovery 90 /		(0.11) Recovery 81
6 h	0.5	2.1	2 8	133	2.1	2.7	4 8	3 9
9 h	0.6	2.0	1 4	70	2.2	2.1	5 3	2 4
12 h	0.8	1 9	1 5	79	_4	5 3	1 9	0 50
24 h	1 0	1 6	1.2	75	2 6	6.4	0 6	0 42
36 h	1 6	1 0	0 9	90				0 70
48 h	1 9	0.7	0 7	100				0.16
72 h	2.4	0.2	0.28					0 02
120 h	2.6	0.0	0.12					0.02
168 h	2.4	0.2	0 10					0.03

the NA loss. In the next two days there was an almost perfect stoichiometric replacement of NA by metaraminol.

After injection of αMMT there was a drop also in the levels of 5HT and DA in the rat brain (table 1). The 5HT concentration was maximally lowered or about 50 / of the normal at 3 hours. The depletion of DA was between those of 5HT and NA, both in time and extent. At no time did the observed quantity of α methylmetatyramine amount to the total loss of DA and 5HT Traces of α-methylmetatyramine could still be detected 48 hours after the injection of αMMT

The smallest dose of intraperitoneally administered DL-αMMT producing a marked reduction of NA concentration in brain and heart was 40 mg/kg. There was a NA deficit in brain of about 1 1 nmol/g 24 hours after such an injection and concomitantly a metaraminol level of 0 7 nmol/g.

2. *Amine levels in the rat heart after αMMT*

The level of NA in the rat heart behaved essentially like that in the brain after intraperitoneal injection of 400 mg/kg DL-αMMT There was a maximal drop of approximately 80 / in the first 12 hours and thereafter a gradual rise for about three days (table 2). In the heart also the missing NA seemed to be roughly balanced by the recovered metaraminol. As in the brain, there was an excess of metaraminol compared to the loss of NA at 6 hours and thereafter a small deficit, which seemed to get smaller in the course of time. At 6 and 9 hours rather large amounts of α-methylmetatyramine also were observed in the heart.

Table 2

Levels of noradrenaline (NA), metaraminol (MA) and α-methylmetatyramine (αMMTA) in the rat heart at various times after 400 mg/kg intraperitoneal DL-α-methyl metatyrosine. The normal NA value is an average ± s.m. from three experiments. All values are corrected for recovery and tissue blank (in bracket).

Time after injection	NA in nmol/g	NA deficit in nmol/g	MA in nmol/g	MA in % of the NA deficit	MMTA in nmol/g	αMMTA in % of the NA deficit
0	5 0 0 23 Recovery = 97		(0.33) Recovery = 83 %		(0.19) Recovery = 89 %	
6 h	1 5	3 5	4 3	123	3.3	94
9 h	1 3	3 7	2.1	57	1.3	35
12 h	1 0	4 0	2.9	73	0.06	
4 h	0 9	4 1	3 4	83	0.06	
36 h	2 2	2 8	–7	96	0.08	
48 h	2	8	–7	94	0 09	
72 h	4.6	0 4	1.3		0.04	–
120 h	5.1	0.1	0.30		0 02	
168 h	4.9	0 1	0 06		0 07	

0.225 respectively The samples obtained from tissues of untreated animals had low absorbances. The corresponding amounts of α-methylmetatyramines were calculated and are shown in brackets in the tables. The values of these tissue blanks were always subtracted from those of the samples. The recoveries of 1–2 μg of amines added to the tissues before homogenization are also given in the tables. All values were corrected accordingly In the rabbit experiments it was assumed that the recoveries were equal from the cerebellum and the other examined tissues.

The NA was determined as described by BERTLER, CARLSSON & ROSENGREN (1958) and the DA by the method of CARLSSON & WALDECK (1958) as modified by CARLSSON & LINDQVIST (1962) The 5HT was assayed on measured portions of the tissue extract as described by BERTLER (1961) and CARLSSON & LINDQVIST (1962)

Results

1 *Amine levels in the rat brain after αMMT*

The time course of the changes in brain amine levels was followed in rats after intraperitoneal injection of αMMT at a dose of 400 mg/kg (DL form) The NA concentration dropped by some 80 / within 6 hours and remained low then rising slowly for several days (table 1) Metaraminol occurred in the rat brain as long as the NA level was reduced and the amount of *missing* NA *roughly* corresponded to the amount of metaraminol recovered However except at 6 hours the amount of metaraminol during the first day seemed to be somewhat smaller than

Table 1

Levels of noradrenaline (NA) metaraminol (MA) 5-hydroxytryptamine (5HT), dopamine (DA) and α methylmetatyramine (αMMTA) in the rat brain at various times after 400 mg/kg intraperitoneal DL-α-methyl metatyrosine. The normal values are averages ± s.e.m. from three experiments on nine rats. All values are corrected for recovery and tissue blank (in bracket)

Time after injection	NA in nmol/g	NA deficit in nmol/g	MA in nmol/g	MA in % of the NA deficit	5HT in nmol/g	DA in nmol/g	5HT + DA deficit in nmol/g	αMMTA nmol/g
0	2.6 ± 0.11 Recovery = 97		(0 16) Recovery = 78		2.6 0.0[illegible] Recovery 78	7 0 0 48 Recovery ~ 90		(0.11) Recovery = 81
6 h	0.5	2.1	2 8	133	2.1	2.7	4 8	3.9
9 h	0.6	2.0	1 4	70	2.2	2.1	5 3	[illegible]4
12 h	0 8	1 9	1 5	79	[illegible]4	5 3	1 9	0.50
24 h	1.0	1 6	1 2	75	6	6 4	0.6	0.42
36 h	1 6	1.0	0.9	90				0.20
48 h	1.9	0 7	0.7	100				0.16
72 h	2.4	0.2	0.[illegible]8					0.02
120 h	[illegible]6	0.0	0.1					0.0[illegible]
168 h	2.4	0.2	0.10			–	–	0.03

in the two brain parts were summed, about 92 % of the depleted NA was balanced by α methylmetatyramines.

Small quantities of metaraminol and α-methylmetatyramine were observed in the heart and the corpora striata, respectively although the concentrations of the naturally occurring amine analogues in these sites virtually had returned to normal

4 Amine levels in the guinea-pig heart after metaraminol

UDENFRIEND and coworkers have used the guinea pig heart in their studies on the mechanism of the NA depletion by αMMT and metaraminol (UDENFRIEND CONNAMACHER & HESS 1961 UDENFRIEND & ZALTZMAN-NIRENBERG 1962). For that reason, and on account of the high NA content of this organ, a few experiments were made on it. *It was found that, after intraperitoneal injection of 0.50 mg/kg (3 0 nmol/g) metara* minol (laevo-form, bitartrate, calculated as base), the heart was severely depleted of NA but was also rapidly replenished (table 4) At 12 and 24 hours metaraminol was detected in amounts corresponding to about 75 % of the NA loss. A small quantity of metaraminol was left even at 36 hours when the level of NA was virtually normal.

Table 4

Levels of noradrenaline (NA) and metaraminol (MA) in the guinea-pig heart at various times after intraperitoneal injection of metaraminol. The normal NA value is an average ± s.m. from three experiments. All values are corrected for recovery and tissue blank (in bracket).

Dose and time	NA in nmol/g	NA deficit in nmol/g	MA in nmol/g	MA in % of the NA deficit
0	13.0 ± 0.60 Recovery = 97 %		(0.20) Recovery = 75 %	
0.50 mg/kg, 12 h	2.6	10.4	7.8	75
0.50 mg/kg, 24 h	4.4	8.6	6.7	78
0.50 mg/kg, 36 h	13.0	0.0	1 1	
0.15 mg/kg, 16 h	8.1	4 9	4.5	92

UDENFRIEND & ZALTZMAN-NIRENBERG (1962) observed that metaraminol injected intraperitoneally at a dose of 0.9 nmol/g (0.15 mg/kg) produced in 16 hours a loss of about 5 nmol/g NA in the guinea pig. This was confirmed by us (table 4). A content of metaraminol equivalent in amount to the missing NA was detected. Thus, the level of metaraminol found in the *guinea-pig heart was about five times higher than that of the* administered amine evenly distributed throughout the whole body

After injection of 40 mg/kg DL-αMMT intraperitoneally there was a loss of about 3 0 nmol/g NA in the heart at 24 hours, whereas the metaraminol concentration at the same time was 2.5 nmol/g.

3 *Amine levels in rabbit tissues 7 days after 100 mg/kg αMMT*

An experiment was carried out with rabbits because the group in BRODIE's laboratory have used mainly this species in their studies on the mechanism of NA depletion by αMMT (GESSA *et al* 1962b) In agreement with these investigators it was found that the NA level in the brain-stem as in the cerebral hemispheres was markedly depressed 7 days after an intravenous injection of 100 mg/kg DL-αMMT (table 3) GESSA *et al.* (1962b) have shown that the NA concentration in the brain-stem begins to rise after this interval At 7 days the DA level in the corpus striatum and the NA level in the heart were approximately re-established

Table 3

Levels of dopamine (DA) α methylmetatyramine (αMMTA) noradrenaline (NA) and metaraminol (MA) in different parts of the brain and in the heart of rabbits, untreated or treated with 100 mg/kg intravenous DL-α methyl metatyrosine (αMMT) 7 days before. All values are corrected for recovery and tissue blank (in bracket).

	Untreated				αMMT 100 mg/kg i.v 7 days				
	DA in nmol/g	αMMTA in nmol/g	NA in nmol/g	MA in nmol/g	DA in nmol/g	αMMTA in nmol/g	NA in nmol/g	MA in nmol/g	MA + αMMTA [illegible]
Corpora striata	16.6	(0.2)	–		13 1	1 3	–		
Cerebral hemispheres	–	(0.1)	1 5	(0 1)		0 1	0.6	1 2	144
Brain stem	–	(0.1)	4 2	(0.1)		0.3	0 7	2.3	74
Cerebellum	–	Recovery = 87		Recovery = 71	–		–		
Heart	–	(0 1)	8 4	(0 2)		0 0	9 1	0.4	–

Table 3 shows that in the brain-stem and in the cerebral hemispheres there were α methylmetatyramines in amounts approximately corresponding to the amounts of missing NA Most of the α methylated amines was metaraminol. The apparent excess of α methylmetatyramines in the cerebral hemispheres and the corresponding deficit in the brain stem compared to the NA loss may be due to differences in the division of the brains from the untreated and the treated animals. If the amine amounts

more, after administration of αMMT there is an almost perfect stoichiometric replacement of the missing NA by metaraminol. The same phenomenon occurs also after administration of metaraminol.

The greatest difference between the conduct of metaraminol determinations in this laboratory and by UDENFRIEND's and BRODIE's groups seems to be in the extraction procedure. Whereas we always use 0.4 N perchloric acid, they extract the amines from the tissues by such an agent as weak as 0.1 N-HCl. Further we homogenize by means of a rotating metal knife ("Ultra-Turrax") and not with a glass or plastic pestle. The glass homogenizer often produces insufficient homogenization, especially of the heart and tough peripheral organs. Much less metaraminol than corresponded to the lost NA was found in the rat heart and brain 24 and 48 hours after the injection of αMMT (400 mg/kg i. p.) when no re-extraction with 0.4 N perchloric acid but otherwise the same determination as described here was carried out (results not shown). After extraction with 0.1 N-HCl and glass homogenization, hardly any metaraminol at all was detected, in agreement with the findings of GESSA *et al* (1962a & b). Nor was metaraminol found in the guinea-pig heart in the same amounts as with the assay method described in this paper (*cf* UDENFRIEND & ZALTZMAN-NIRENBERG 1962). Thus, it seems that metaraminol is much more firmly fixed to the tissues than the naturally occurring monoamines. This *in vitro* finding may possibly contribute to explaining the long half-life of metaraminol *in vivo* in addition to the ineffectiveness of the enzymes monoamine oxidase and catechol-O-methyl transferase in the degradation of this amine.

Whereas the level of 5HT in the rat brain had apparently returned to normal already at 6 hours, that of DA was depressed for about 24 hours after injection of αMMT. The analogue of DA formed, *i. e.* α-methylmetatyramine, was never found in amounts corresponding to the missing DA. The deficit may be even larger since part of the α-methylmetatyramine is probably situated in NA neurons at 6 and 9 hours (*cf* the heart). Neither did the quantities of metaraminol found in the rat brain and heart at 9 1? and 24 hours entirely equal the NA loss. The deficits observed may be due to incomplete recoveries of firmly tissue-bound α-methylmetatyramines, but there is at least one further explanation. It has been observed that α-methyl DOPA gives an almost parallel depression in concentrations of 5HT and its acid degradation product 5-hydroxyindolylacetic acid in brain (SHARMAN & SMITH 1962, ROOS & WERDINIUS 1963, PLETSCHER, BURKARD & GEY 1964). These results favour an inhibited synthesis. As more potent decarboxylase inhibitors do not cause a drop in 5HT, it was suggested that α-methyl DOPA inhibits the first step in the synthesis of 5HT, *i. e.* the hydroxylation of tryptophan in the 5-position (ROOS &

Discussion

The earlier concept that monoamine depletion by the α-methylated amino acids is entirely due to decarboxylase inhibition became untenable when it was found that the synthesis rate of the monoamines is about normal during the greater part of the long period with depressed NA levels after αMMT (HESS *et al* 1961 COSTA *et al* 1962a) The former investigators also gave evidence for the view that the prolonged effect on NA concentration is caused by impairment of the tissues ability to accumulate and hold NA An explanation of the αMMT action on NA storage was presented by CARLSSON & LINDQVIST (1962) They observed by paper chromatography that after administration of αMMT there is in the tissues a metaraminol content approximately equivalent to that of the missing NA. After injections of α methyl DOPA the lost NA has instead been roughly replaced by α methyl NA (*cf* MAÎTRE & STAEHELIN 1963 MUSCHOLL & MAÎTRE 1963) From these findings it was concluded that the drop in NA is at least mainly due to displacement by the amine analogues formed. With the use of potent decarboxylase inhibitors, such as α methyl DOPA hydrazine (UDENFRIEND CONNAMACHER & HESS 1961) or certain metahydroxybenzylhydrazines and metahydroxybenzyloxy amines (DRAIN *et al* 1962 BRODIE *et al* 1962) it was soon proved that the prolonged NA depletion after αMMT is mediated by the decarboxylation products (UDENFRIEND & ZALTZMAN-NIRENBERG 1962 GESSA *et al* 1962a) However these investigators failed to detect α methylmetatyramines in amounts balancing the NA loss, especially more than 24 hours after the injection of αMMT or metaraminol (UDENFRIEND & ZALTZMAN-NIRENBERG 1962 GESSA *et al* 1962b) On account of the absence of stoichiometric equivalence it was suggested that the α methylmetatyramines act in the same way as reserpine on the NA stores. As is well known, one reserpine molecule can eliminate several hundreds of monoamine molecules (CARLSSON, SHORE & BRODIE 1957) Many of the experiments reported by UDENFRIEND s and BRODIE s groups (see also UDENFRIEND CONNAMACHER & HESS 1961 COSTA *et al* 1962b BRODIE & COSTA 1962) have been repeated in this laboratory In all instances metaraminol has been found by paper chromatography (CARLSSON 1964a) in the rat brain and heart 6 hours after 400 mg/kg intraperitoneal αMMT in the rabbit brain stem 7 days after 100 mg/kg intravenous αMMT and in the guinea-pig heart 24 hours after 0 2 mg/kg intraperitoneal metaraminol. The results obtained by this semiquantitative method of determination have been wholly confirmed in our study In contrast to the findings of UDENFRIEND & ZALTZMAN-NIRENBERG (1962) as well as those of GESSA *et al* (1962b) the results given in this paper show that at 36 hours, and

more, after administration of αMMT there is an almost perfect stoichiometric replacement of the missing NA by metaraminol. The same phenomenon occurs also after administration of metaraminol.

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WERDINIUS 1963) This view is strengthened by the finding that α-methyl DOPA after a decarboxylase inhibitor still produces a drop in 5HT and DA and the long lasting lowering in NA levels is abolished (CARLSSON 1964a) Later this hypothesis has received additional support by the observation that α methyl DOPA inhibits liver tryptophan hydroxylase, which may be related to though not identical with, the corresponding brain enzyme (BURKARD GEY & PLETSCHER 1964) These investigators found that αMMT is a weak inhibitor of the liver enzyme *in vitro* The large loss of DA and, at 9–24 hours, also of NA compared to the amounts of α methyl metatyramine and metaraminol detected, respectively may be due to inhibition of synthesis The inhibition seemed to last for as long as αMMT has been found at high concentration in the tissues *i e* for about six hours (UDENFRIEND & ZALTZMAN-NIRENBERG 1962 GESSA *et al* 1962a)

We have observed weak sedation and miosis of short duration after administering αMMT to cats, but only after repeated injections (ANDÉN & MAGNUSSON unpublished results) It was, however much easier to detect the enhanced reaction after a repeated dose when α methyl DOPA was used This was seen both in cats (sedation miosis) and rats (sedation, ptosis) The second injection was given 24 hours after the first one, when the appearance of the animals was virtually normal It has been observed that αMMT itself can produce sedation and counteract hypertension in man, but these effects are potentiated much by pretreatment with α methyl DOPA (HORWITZ & SJOERDSMA 1964) The mechanism of the enhanced action after a priming dose of an α methylated amino acid is now being investigated It will then be of interest to compare α methyl DOPA and αMMT since both produce a long lasting lowering of NA levels but differ in sedative and antihypertensive effects.

Besides the differences between reserpine and αMMT mentioned in the introduction there is at least one other it is much easier to obtain a virtually complete depletion of tissue monoamines by reserpine In studies on the functional significance of NA displacement by metaraminol in cats and rats, the most severe depletion of NA was obtained after two injections of 400 mg/kg intraperitoneal αMMT and 0 2 mg/kg intra venous (—)-metaraminol 48 24 and 4 hours before the experiment (ANDÉN & MAGNUSSON 1964) However even after this intense treatment, about 5% of the NA normally occurring remained in the organs examined (heart, spleen, iris, nictitating membrane) The function of the sympathetic nerves appeared unimpaired after displacement of about 95% NA by the approximately three times less active metaraminol It may be argued that the apparently normal sympathetic tone was due to the accumulation of large amounts of α methylmetatyramines However determinations of

amines by the methods described in this paper were made in a few cases and showed an acceptable agreement between found metaraminol and missing NA. We have also made functional analyses of the sympathetic nervous system when the NA displacement has been smaller than 95% on account of a less intense treatment (for example αMMT 200 + 250 mg/kg subcutaneously 48 and 24 hours before, or αMMT 200 mg/kg intraperitoneally daily for five days or metaraminol 2.5 mg/kg intraperitoneally twice daily for four days). At no instance has it been possible to demonstrate a deterioration in adrenergic transmission. Similar observations on dogs have been reported by STONE *et al.* (1962), although with the doses used in their experiments the NA had dropped only by some 50%. Thus, it seems as if the larger part of the NA store in the nerve terminals is not essential for normal transmission. Experiments are in progress to elucidate the mechanism behind this phenomenon.

Summary

The noradrenaline levels in the rat heart and brain were depressed for several days after injection of α-methylmetatyrosine (400 mg/kg intraperitoneally). The noradrenaline was replaced almost stoichiometrically by the metabolite metaraminol (α-methyl-β-hydroxymetatyramine) 36 hours or more after the administration. At the earlier intervals there was a small deficit of metaraminol and a marked one of α-methylmetatyramine compared to the losses of noradrenaline and dopamine, respectively which may have been due to inhibition of synthesis. The missing noradrenaline was approximately replaced by stoichiometric amounts of metaraminol in the rabbit brain also (100 mg/kg α-methylmetatyrosine intravenously 7 days), in the guinea-pig heart after injection of metaraminol (0.15–0.50 mg/kg intraperitoneally) and in the rat brain and heart after a smaller dose of α-methyl metatyrosine (40 mg/kg intraperitoneally). The metaraminol appeared to be so firmly fixed to the tissues that it was necessary to use large volumes of 0.4 N perchloric acid twice and metal homogenization to extract it. The displacement of noradrenaline by metaraminol did not seem to impair adrenergic transmission. It may however enhance the functional effect caused by α-methylated amino acids.

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On the Relation of Tryptaminic and Serotonergic Mechanisms to Amphetamine Induced Abnormal Behaviour

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A previous paper (RANDRUP MUNKVAD & UDSEN 1963) described a certain kind of abnormal behaviour in rats caused by amphetamine injections This behaviour was distinctly stereotyped and could not be counteracted by several powerful adrenergic blocking agents, even in large doses. It was also found that similar behaviour could be produced by the tryptamine analogue of amphetamine, α methyltryptamine.

These findings bring to mind the hypothesis of Vane (VANE 1960 GELDER & VANE 1962) who has suggested that amphetamine may act upon tryptamine or 5-hydroxytryptamine (serotonin) receptors in the central nervous system. In this paper we describe our investigations into this possibility Amphetamine and α methyltryptamine were injected along with various substances having strong anti tryptamine and anti-serotonin activity Further we tested a number of tryptamine derivatives that have been reported to antagonize hyperactivity induced by amphetamine and to exert other sedative effects.

Methods

Our technique was essentially as described in our previous paper

The animals were highly inbred white male rats from the State Serum Institute, Copenhagen. They were 3–6 months old, weighing 160–350 g. Animals used for a test or cross-over experiment were, however always taken from one consignment from the supplier and had therefore all been in our laboratory for the same period of time, their ages differing by less than two weeks The rats were placed in separate cages made of wire netting. After injection of drugs they were kept under constant observation for four hours or longer by one of the authors (A.R) or by a technician who had had more than a year's training at these experiments.

All injections were given subcutaneously The dose of amphetamine (dexedrine ®)

was always 3 mg/kg as D-amphetamine base. The dose of α-methyltryptamine, 3-(2'-amino-2'methylethyl)-indole, was always 40 mg/kg as hydrochloride of the racemic compound.

The drugs with anti-tryptamine and anti-serotonin activity and their applications are described below

Methysergide (WHO), *deseril* ® (FANCHAMPS, DOEPFNER, WEIDMANN & CERLETTI 1960; DOEPFNER 1962) 150 mg/kg (as the maleinate, dissolved in propyleneglycol-1N H_2SO_4 9 1) were injected 10 min. before amphetamine (2 rats, one died within 24 hrs.) 100 mg/kg were given 10 min. (5 rats) or 30 min. (3 rats) before amphetamine.

Also, 150 mg/kg were given 10 min. before (2 rats) or 4½ hrs. after (2 rats) α-methyltryptamine at the latter moment the stereotyped behaviour was fully developed 100 mg/kg were injected 10 min. (3 rats, 2 died within 24 hrs.) or 30 min. (3 rats, one died within 24 hrs.) before α-methyltryptamine.

Cyproheptadine periactin ® (STONE *et al.* 1961) 150 mg/kg (the hydrochloride dissolved in propyleneglycol were injected 40 min. after amphetamine (2 rats) at this time the stereotyped behaviour was fully developed 100 mg/kg were given 15 min. (3 rats) or 30 min. (3 rats) before amphetamine.

Also 100 mg/kg was given 10 min. (3 rats) or 30 min. (3 rats) before α-methyltryptamine.

2-Bromo-lysergide BOL – 148, (BERDE, DOEPFNER & CERLETTI 1960) 30 mg/kg (the bitartrate dissolved in 0.025 N HCl) were injected 10 min. before (3 rats), 30 min. before (6 rats) or about 45 min. after (2 rats) amphetamine, 10 mg/kg were given 10 min. before amphetamine (10 rats).

Benanserine, BAS, see fig. 1 (WOOLLEY VAN WINKLE & SHAW 1957) 100 mg/kg (the hydrochloride dissolved in propyleneglycol) were injected 15 min. before (5 rats), 30 min. before (7 rats) or 30 min. after (2 rats) amphetamine 9 of these 14 animals died within 24 hrs.

N N-dimethyl-benanserine, BAB, (SHAW & WOLLEY 1956) 100 mg/kg (the hydrochloride, dissolved in propyleneglycol-1N H_2SO_4 9 1) were injected 15 min. (5 rats) or 30 min. (7 rats, 2 died within 24 hrs.) before amphetamine.

Of the tryptamine derivatives listed in table 3 N,N-diethyltryptamine (the bioxalate, dissolved in saline) was injected from 20 min. before to 30 min. after amphetamine, 2-phenyl-N,N-diethyltryptamine (the hydrochloride in propylene glycol) was given 10 min. before, 5-hydroxytryptamine (the hydrogen oxalate, from Regis, U.S.A. in water) simultaneously with, IN 461 (the hydrochloride in water) 35 min. before or 25 min. after amphetamine. Oxypertine (in propylene glycol) was given 1 hour before amphetamine.

All drugs except amphetamine and oxypertine (the latter dissolves slowly) were dissolved immediately before use. *The concentrations of the solutions were adjusted* so that the volumes injected were always less than 7.5 ml/kg.

With each drug *preliminary experiments were made, usually on only a few animals*, and final experiments with 3 to 10 animals for each drug combination. Cross-over procedure was used when suitable. The animals used for the final experiments had never been treated with any drug before. Since the results of the preliminary and the final experiments never differed, the number of rats given above and in the tables is those used in both types of experiments.

Counting of grooming spells.

Rats show three forms of grooming: grooming of the head with the forelimbs, of the body with the nose and mouth and of the neck and body with the hind limbs. Each

of these forms was counted separately so that if a rat shifted from one form into another it was counted as showing a new grooming spell. Such shifts often happened. Six rats could be followed by one observer another observer was present, recording the rest of the behaviour

Results

The abnormal behaviour induced in rats by 3 mg/kg s.c. of D-amphetamine was described in our previous paper From about 30 min. to about 2 hrs. after the injection the behavioural features described below were seen in all rats (155 rats so far except one that only displayed the constant sniffing)

A. Constant and vigorous sniffing, which covers only a small area, about two square inches, of the cage floor or the lower part of one of the walls. Sniffing is accompanied by movements of the mouth and licking or biting the wire netting of the cage.

B Locomotion is only seen in a few rats and these always move backwards

C. Pressing of body against cage walls

From about 20 to 30 min. and again about 2 hrs. after the injection, forward locomotion is seen along with constant sniffing of the cage without movements of the mouth From 2–5 hrs. after the injection normal activities (grooming, walking, eating and drinking) gradually replace the stereotyped activity and the animals finally fall asleep Closely similar behaviour is elicited by 40 mg/kg s c. of α methyltryptamine, but with this drug the abnormal behaviour begins later and lasts longer

The effect of anti tryptamine and anti serotonin substances

Of the five substances with anti tryptamine and anti serotonin activity only BOL 148 is able to counteract the stereotyped behaviour (see table 1 and 2) It is important to note that the effective dose of BOL 148 is much lower than the doses of the other drugs applied BOL 148 renders the animals quiet, and the effect is much like that of the tranquillizers, chlor promazine and haloperidol described in our first paper

Although they lacked effect on the stereotyped behaviour several of the drugs showed some other effects on the rats (see table 1 and 2) which were observed while the stereotyped activity was being performed. It may also be seen from table 1 that, though the smallest only partly effective dose of BOL shortened the stereotyped activity none of the other drugs did so indeed, two of them caused marked prolongation.

Each of the drugs methysergid, cyproheptadine, BAS and BAB were given at a dose of 100 mg/kg to 3 rats with a placebo instead of ampheta

Table 1

Effects of substances with anti-tryptamine and anti-serotonin properties upon rats injected with 3 mg/kg s.c. of D-amphetamine.

Substance	Dose mg/kg s.c.	Inhibition of stereotype activity[2])	Other effects on rats treated with amphetamine.
Methysergide	150 (2)[1]) 100 (8)	0/2 0/8	Body not pressed against cage wall. Forward movement of some rats. No shortening of stereotyped behaviour[3]).
Cyproheptadine	150 (2) 100 (6)	0/2 0/6	Forward movement, paws of forelegs sometimes placed on walls. Lower lip retracted. Prolongation of stereotyped behaviour[4]).
2-bromo-lysergide	30 (11) 10 (10)	11/11 5/10	Quiet. Forward movement. Shortening of stereotyped behaviour[5]).
Benanserine	100 (14)	0/14	Sedation[6]).
(N,N-dimethyl-benanserine)	100 (12)	0/12	Prolongation of stereotyped activity[7]).

[1]) The figures in parentheses show the numbers of animals; for details of injections and survival, see under "Methods".
[2]) Inhibition means that constant sniffing, licking or biting is not seen at all for 0–4 hrs. after amphetamine.
[3]) Much sniffing, although not constant in all rats 7 hrs. after amphetamine, when 3 rats given placebo + amphetamine were all sleeping.
[4]) Continuous stereotyped behaviour 8 hrs. after amphetamine, when 3 rats given placebo + amphetamine were all sleeping.
[5]) 8 of the 10 animals in cross-over experiment with placebo. The rats that did show continuous sniffing with BOL began later and stopped earlier than with placebo. The difference was clear 20–40 min. and again 2–2½ hrs. after amphetamine.
[6]) 8 out of 10 rats fell down from vertical wire netting, 3 of these remained lying on their backs on the table for more than 10 secs.
[7]) 7 hrs. after amphetamine 9 out of 10 rats were still showing constant stereotyped behaviour; 5 rats given placebo + amphetamine were all sleeping.

mine. With the three former drugs the behaviour was not materially different from that of untreated animals: they slept most of the time or otherwise performed normal activities, such as grooming, eating and drinking. With BAB the rats stayed awake: they were quiet in the cage, but appeared nervous when handled. Thus, stereotyped activity was not observed with any of these drugs.

We also considered the possibility that some of the drugs might increase the effect of amphetamine. This possibility was checked and disproved for deseril and cyproheptadine by experiments in which the two drugs were given along with smaller doses of amphetamine (4 rats with each of the drugs).

of these forms was counted separately so that if a rat shifted from one form into another it was counted as showing a new grooming spell Such shifts often happened. Six rats could be followed by one observer another observer was present, recording the rest of the behaviour

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Each of the drugs methysergid, cyproheptadine BAS and BAB were given at a dose of 100 mg/kg to 3 rats with a placebo instead of ampheta-

Table 3

Effect of tryptamine derivatives with reported tranquillizing effect (see text) upon rats injected with 3 mg/kg s.c. of D-amphetamine.

Substances	Doses mg/kg s.c.	Inhibition of stereotyped behaviour[2])	Other effects on amphetamine treated rats.
N,N-diethyl-tryptamine	1 10 (8)[1]) 20–48 (4)	0/8 0/4	2 rats died within 24 hrs.
2-phenyl-N,N-diethyl-tryptamine	50–100 (4) 200 (7) 300 (2)	0/4 5/7[3]) 1/2	Forward movement, all survived more than 24 hrs.
5-Hydroxy-tryptamine	7 14 (8) 70–140 (5)	0/8 0/5	Ears and paws extremely red. 3 rats died within 24 hrs.
IN 461)	400 (4)	0/4	
Oxypertine[5])	14 (8) 20 (5)	8/8 5/5	Grooming[4]) and forward movement.

[1–2]) See table 1

[3]) See fig. 1

[4]) See table 4.

[5] J. Buus Lassen, Ferrosan, Copenhagen, found that 40 mg/kg s.c. given simultaneously with 1,8 mg/kg s.c. of D-amphetamine inhibited constant sniffing in 4/4 rats.

tine, inhibited the stereotyped reactions caused by amphetamine, as shown in table 3 As we did not make quantitative measurements of general activity it is possible that the other substances reduce hyperactivity without affecting the stereotyped behaviour The same might apply to some of the adrenergic blocking agents studied earlier (RANDRUP MUNKVAD & UDSEN 1963)

Oxypertine was the most interesting substance of this groups. A dose of 14 mg/kg inhibited the stereotyped behavior pattern almost completely only during a period from 30 to 60 minutes after the injection of amphetamine did some of the animals display any appreciable amount of sniffing. Even these animals, however sometimes paused with the nose off the wire netting and never indulged in constant sniffing. Further oxypertine increased grooming activity (see table 4), and the rats also showed much movement in a forward direction. Oxypertine, then, did not render the rats as quiet as we have seen previously with chlorpromazine or other major tranquillizers. With a larger dose, 20 mg/kg, sniffing was almost completely absent, but so was movement, though grooming was still seen (5 rats).

Table 2

The effects of methysergide and cyproheptadine upon rats injected with 40 mg/kg s.c. of DL α methyltryptamine. (3-(2-amino-2 methylethyl)-indole).

Substances	Doses	Inhibition of stereotype activity[2])	Other effects on rats treated with α methyltryptamine.
Methysergide	150 (4)[1])	0/4	Earlier onset of walking and stereotyped behaviour[3]).
	100 (6)	0/6	
Cyproheptadine	100 (6)	0/6	Forward locomotion, paws of forelegs sometimes placed on walls. Lower lip retracted.

[1,2]) See table 1

[3]) Compared with 3 rats given placebo +α-methyltryptamine and earlier experience with α methyltryptamine.

Effect of some tryptamines with tranquilli ing action

For further investigation of the problem we chose some tryptamine derivatives that have been reported in the literature to antagonize hyper activity of mice induced by amphetamine or methamphetamine These include N N-diethyltryptamine and its 2 phenyl derivative (PFEIFFER, SATORY & PATAKY 1961 BORSY LÉNÁRD & CZISMADIA 1961), serotonin (KOBINGER 1958) and IN 461 (see fig. 1 MIRSKY WHITE & O DELL 1959). In addition we tried oxypertine (see fig. 1) which is reported to have the same central effects as chlorpromazine (WYLIE & ARCHER 1962 ARCHER *et al* 1962)

Of these substances only 2 phenyl N N-diethyltryptamine and oxyper

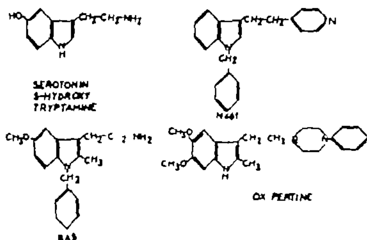


Fig. 1

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Substances	Doses mg/kg s.c.	Inhibition of stereotyped beha iour[2]	Other effects on amphetamine treated rats.
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2-phenyl-N,N-diethyl-tryptamine	50–100 (4) 200 (7) 300 (2)	0/4 5/7[3] 1/2	Forward mo ement, all survived more than 24 hrs.
5-Hydroxy-tryptamine	7–14 (8) 70–140 (5)	0/8 0/5	Ears and paws extremely red. 3 rats died within 24 hrs.
IN 461[1]	400 (4)	0/4	
Oxypertine[3])	14 (8) 20 (5)	8/8 5/5	Grooming[4]) and forward movement.

1–3) See table 1

2) See fig. 1

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Cross-over experiment with 2 × 3 animals, 2 weeks between the two experimental days. In 30 min.–1 hour after amphetamine, the animals showed maximum (though not constant) sniffing when treated with oxypertine. When treated with placebo, all six animals sniffed and licked constantly during the whole period 1–2 hours after amphetamine. See also text.

Period after inj. of amphetamine		30 min.–1 h.	1 h.–2 hrs.
No. of grooming spells	Oxypertine + amph.	3.3 ± 2.0[1])	12.7 ± 4.1
	Placebo + amph.	1.0 ± 1.0	1.0 ± 0.8
Difference		2.3 ± 1.0[2])	11.7 ± 3.7
Significance of Difference		Not signif.	P = 0.025

[1]) Standard error of the mean.

[2]) In order to eliminate individual variation from the statistical evaluation, the differences between oxypertine and placebo for each animal were first calculated (they were all positive in both periods) and the means of these differences then entered in the table.

Discussion

For evaluating the negative results with the serotonin and tryptamine antagonists (tables 1 and 2) it is important to know whether these substances are able to cross the blood brain barrier and thereby to have any effect on the central nervous system. Methysergide has been demonstrated in the brain of rats after intravenous injection (DOEPFNER 1962) and central effects of peripherally applied methysergide, cyproheptadine and BAB have been reported repeatedly (CORNE, PICKERING & WARNER 1963, GARATTINI & VALZELLI 1962, VANE *et al.* 1961, KÄRJA, KARKI & TALA 1961, FANCHAMPS *et al.* 1960, STONE *et al.* 1961 see p. 83, MERCK SHARP & DOHME 1962, JACOB & LAFILLE 1963, LAVENSTEIN *et al.* 1963, SHAW & WOOLLEY 1956, RINALDI 1958). With BAS the evidence is more doubtful, but the sedative effect observed in our experiments has also been reported in the literature as well as EEG effects (WOOLLEY, VAN WINKLE & SHAW 1957, RUDY *et al.* 1958, RINALDI 1958).

The report of CORNE, PICKERING & WARNER (1963) is particularly relevant to our problem. They describe an abnormal head twitch of mice (also observed in rats) elicited by large doses (70–300 mg/kg i.p.) of the serotonin precursor 5-hydroxytryptophane. This activity was antagonized by methysergide, cyproheptadine, BOL and BAB (but not BAS) given subcutaneously, the ED50 being 4.1 mg/kg, 0.3 mg/kg, 3.8 mg/kg and 11 mg/kg, respectively. The effect of the small doses of the antagonists in this head twitch test and the equality between BOL and the other drugs are in

sharp contrast with our results in the stereotyped behaviour test (tables 1 and 2).

The lack of effect of these antagonists on the stereotyped activities can therefore hardly be ascribed to difficulty in penetrating the blood brain barrier

In evaluating our results it must also be considered that in peripheral tissues more than one receptor is sensitive to the action of serotonin and tryptamine (GADDUM & PICARELLI 1957 GYERMEK 1962 WOOLLEY & SHAW 1962 BUNAG & VALASZEK 1962 MARCZYNSKI 1962). In the brain there may also be several receptors present at least it is known that tryptamines elicit many different behavioural and other effects in the central nervous system (WOOLLEY 1962 CORNE *et al.* 1963 WADA *et al* 1963 MARLEY & VANE 1963 KÄRJÄ, KÄRKI & TALA 1961).

The antagonists listed in table 1 as well as dibenzyline and dihydroergotamine (see RANDRUP MUNKVAD & UDSEN), are all known to block the so-called D-receptors of serotonin (GADDUM & PICARELLI 1957 GYERMEK 1962). It has also been shown that methysergide and cyproheptadine inhibit some effects of tryptamine (GARATTINI & VALZELLI 1962 ERLE 1963 VANE *et al.* 1961).

As to the so-called nervous receptors of serotonin (M-type of Gaddum, B-type of Gyermek), specific antagonists have recently been developed (GYERMEK 1962), but these have not been available in sufficient quantity for our experiments, and information about their passage through the blood-brain barrier and possible effects on the central nervous system is not yet available.

It should, however be noted that GYERMEK & BINDLER (1962), while studying a series of tryptamine derivatives, found that lack of a hydroxyl group in the 5-position, as well as substitution of a methyl group in the α-position of the side chain, strongly reduced the affinity for the B-receptors. It is therefore unlikely that α-methyltryptamine, whose molecule has both of these features, should exert its behavioural effects via the B-receptors.

Atropine blocks the M-receptors (GADDUM & PICARELLI 1957) and also antagonizes some behavioural and EEG effects of 5-hydroxytryptophane (WADA *et al.* 1963). In some experiments with atropine (1.5–320 mg/kg s.c., 20 rats) we found that it did not inhibit the stereotyped behaviour induced by amphetamine. On the other hand the EEG desynchronization induced by amphetamine is reported to be effectively antagonized by atropine (BRADLEY & ELKES 1957). Therefore these results also indicate that desynchronization or "activation" of the EEG corticogram is not a necessary prerequisite for the stereotyped behaviour

The duplication of the amphetamine stereotyped behaviour by α-

Table 4

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Period after inj. of amphetamine		30 min.–1 h.	1 h. hrs.
No of grooming spells	Oxypertine + amph	3.3 ± 2.0[1]	12.7 ± 4.1
	Placebo + amph.	1 0 ± 1.0	1.0 ± 0.8
Difference		2.3 ± 1 0[2]	11 7 ± 3.7
Significance of Difference		Not signif	P = 0.05

[1]) Standard error of the mean.

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methyltryptamine and the antagonistic actions of oxypertine, 2 phenyl-N,N-diethyltryptamine and BOL show that tryptamines can affect such brain mechanisms as are essential for the behaviour The evidence presented in this paper also indicates, however that the stereotyped behaviour is not elicited *via* any of the tryptamine or serotonin receptors hitherto described. In contrast, the above-mentioned work of CORNE *et al* (1963) indicates that the head twitch studied by these authors is elicited by the action of serotonin on D-receptors in the brain.

Many cases of amphetamine psychosis with schizophreniform symptoms have been reported from the clinic (for references, see RANDRUP MUNKVAD & UDSEN 1963) it was this that originally elicited our interest in the amphetamine receptors of the central nervous system. Though well aware of the differences between rat and man we suggest that it might be interesting for both clinical and theoretical reasons to find a highly active specific antagonist against the stereotyped behaviour of the rats. According to the results reported in this and previous papers, there is no promise in a search for such an antagonist among compounds that inhibit the known tryptamine or adrenergic receptors. Substitution of a piperazine ring for the amino group in certain classes of amines appears to give a better chance. This is illustrated in fig. 1 in which it is shown that, except for the piperazine ring, oxypertine is closely related chemically to both IN 461 and BAS The anti-amphetamine effects of these substances are, however different. Particularly it should be noted that, whereas BAS sedates the rats without inhibiting the stereotyped activities, oxypertine inhibits them and at the same time *increases* some normal activities (see tables 1 3 and 4) A similar effect was observed with two piperazine derivatives of phenothiazine (perphenazine and thioproperazine) but not all piperazines have this action the substance KSW 3019 a piperazine derivative of chlorphencyclane, KSW 788 (SCHAUMANN & KURBJUWEIT 1961) was found by us to *elicit* a behaviour much like that induced by amphetamine (20–40 mg/kg s c. 4 rats)

Summary

The abnormal stereotyped behaviour of rats induced by amphetamine can be duplicated or antagonized by different derivatives of tryptamine. The strong serotonin inhibitors methysergide and cyproheptadine have however no effect on the stereotyped activities, and this together with other evidence indicates that the hitherto known receptors for serotonin and tryptamine are not involved

Suggestions are made for possible ways of finding specific antagonists against this behavioural effect of amphetamine.

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Some Observations on Adrenergic Connections between Mesencephalon and Cerebral Hemispheres

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In a former investigation (Bertler *et al.* 1963) it has been demonstrated that destruction of the cerebral cortex or the thalamus did not produce any significant alteration in the dopamine level of the caudate nucleus. In continuance of this work the influence of mesencephalic lesions on the catecholamine contents of the hemispheres and the diencephalon has been studied, particularly with the object of localizing dopamine in the striata. In addition, some observations on the localization of adrenergic structures in these areas are reported.

Material and methods

Brain material from pigeon and rabbit was used. Chemical determinations of noradrenaline (NA) and dopamine (DA) in different parts of the normal pigeon brain were performed (see table 1). For fluorescence microscopy the anterior part of the mesencephalon, diencephalon and the adjacent part of the telencephalon were excised in one piece. Further unilateral cross-sections were made in pigeons between the diencephalon and the telencephalon by a sharp instrument directly though the bone. The concentrations of NA and DA were determined fluorimetrically in the parts frontal to the lesion 6–13 days after the operation (table 2). The rabbit material included both normal animals and animals submitted to unilateral electrolytic lesions. The lesions were evoked in the ventral part of the mesencephalon by the stereotaxic instrument of Monnier & Gangloff (1961). The catecholamine contents of the right and left brain halves frontal to the mesencephalon were determined fluorimetrically seven days after the operation. The caudate nuclei and the ventral mesencephalon from normal animals were examined histochemically.

The tissue contents of monoamines were determined by the methods of Bertler *et al.* (1958) and Bertler & Rosengren (1959). The histochemical localization of primary catecholamines was studied by the fluorescence microscopic method of Falck (1962).

Table I

Catecholamine contents of different parts of the pigeon brain. Each sample consisted of pooled parts from three animals.

Brain part	No. of deter	NA, µg/g	DA, µg/g
Telencephalon, except paleostriatum and neostriatum	5	0.27 (0.19–0.34)	0.35 (0.30–0.39)
Paleostriatum + neostriatum	5	0.52 (0.41–0.69)	1 5 (1 2.2)
Diencephalon	5	0.88 (0. 6–1 2)	0.05 (0.00–0.14)
Mesencephalon	5	0.89 (0.78–1 09)	0.05 (0.03–0 09)
Area of mesencephalic fluorescent cells[1])	3	5 2 (3 4–8.5)	0.67 (0 54–0.80)
Cerebellum	5	0.16 (0 13–0 18)	0.01 (0 00–0.04)
Medulla oblongata	3	0.50 (0.30–0 72)	0.03 (0.00–0.10)

[1]) The part of the mesencephalon containing most of the fluorescent cells whose terminals are found in the paleostriatum and neostriatum

Results

As will be seen from table I significant amounts of NA were present in all the parts of the pigeon brain examined, *i e* the telencephalon, the diencephalon, the mesencephalon the cerebellum and the medulla oblongata. The highest concentrations were found in the diencephalon and the mesencephalon in the latter the concentration was especially high in the ventral portion

Most of the DA was found in the telencephalon, where it was mainly localized in a part roughly corresponding to the paleostriatum augmentatum and neostriatum In the other parts examined there was a fairly high concentration of DA in that area of the mesencephalon containing most of the adrenergic nerve-cell bodies sending their axons into the corpus striatum (see below)

By histochemical examination of the pigeon brain a bilaterally symmetric cluster of nerve-cell bodies showing a green fluorescence of moderate intensity was observed in the anterior part of the mesencephalon. This aggregation of cells had an elongated form It appeared at the level of the root of the oculomotor nerve below the nucleus ruber and ran backwards to end laterally in front of nucleus isthmo-opticus. The processes of the cells showed remarkably high fluorescence and could, in contrast to the adrenergic preterminal neurons in the mammalian brain be followed in serial sections A large number of these processes were seen to pass through the nucleus as smooth fibres, to leave it at its most ventral part as a broad bundle merging with the median forebrain bundle. Within the nucleus there also occurred a large number of intensely green fluore-

scent fibres of the "terminal" varicose type having the same appearance as noradrenergic terminals in the mammalian brain (CARLSSON *et al* 1962 & 1964). The preterminal parts of these axons could not be observed in the fluorescence microscope. The smooth fluorescent fibres spread as a fan shape in the sagittal plane laterally in the hypothalamus, to turn at the level of the anterior commissure into the corpus striatum. They could be followed for some distance into the paleostriatum as slender bundles. Of the four subdivisions in the corpus striatum, the paleostriatum primitivum showed a sparse amount of intensely fluorescent varicose fibres having the same appearance as the mammalian noradrenergic terminals. A few scattered fibres of the same type but with somewhat weaker fluorescence, also appeared in the hyperstriatum. An area corresponding to the paleostriatum augmentatum, and to at least the main part of the neostriatum, showed an intense green fluorescence, which at a low magnification seemed to be just as diffuse as that found in the mammalian caudate nucleus (CARLSSON *et al.* 1962). However at higher magnifications the fluorescence could as a rule be clearly seen to be confined to closely packed fibres. These fibres were much finer and more richly varicosed than those seen in other parts of the striatum.

In the pigeons submitted to unilateral sectioning between the diencephalon and telencephalon, the telencephalic concentration of DA was lower in the operated than in the unoperated side in the four animals used (table 2) In two of the experiments there was also a decrease of NA on the operated side.

In the normal rabbit (8 animals) the concentration of NA in the right cerebral hemisphere plus the diencephalon was found to be 0.31 ± 0.06 μg/g (average ± its standard deviation) the corresponding value on the left side was 0.31 ± 0.07 μg/g. The DA concentration in the same brain was 0.51 ± 0.19 μg/g on the right side and 0.47 ± 0.19 μg/g on the left side. In the animals with unilateral mesencephalic lesions, the NA con-

Table 2

Catecholamine content in the cerebral hemisphere of the pigeon after unilateral section between the diencephalon and the telencephalon. Two animals used for each determination.

Exp nr	NA, μg/g		DA, μg/g	
	op. side	unop. side	op. side	unop. side
1	0.27	0.27	0.22	0.45
2	0.11	0.11	0.10	0.18
3	0.13	0.20	0.41	0.58
4	0.05	0.17	0.01	0.24
Mean values	0.14	0.19	0.19	0.36

Table 3

Effect of unilateral lesions in the ventral mesencephalon of the rabbit on the catecholamine content of cerebral hemisphere plus the diencephalon.

Exp. nr	NA μg/g		DA, μg/g	
	op side	unop. side	op. side	unop. side
1	0.13	0.38	0.31	0.57
2	0.10	0.23	0.42	0.45
3	[illegible]	[illegible]	0.20	0.44
4	0.08	0.15	[illegible]	0.42
5	0.06	[illegible]	0.45	0.54
6	0.09	0.11	0.35	0.48
7	0.13	0.27	0.59	0.64
8	0.17	0.27	0.59	0.86
9	0.08	0.20	0.05	0.37
10	0.15	0.18	0.37	0.45
11	0.14	[illegible]	0.51	0.54
Mean values	[illegible]	0.21	0.37	0.52

centration was considerably lower on the operated than on the unoperated side The NA values on the unoperated side were also significantly lower than those for untreated animals (table 3) The DA values obtained in the part frontal to the lesion were significantly lower than those obtained from the corresponding part of the unoperated side (table 3), which did not differ from those found in untreated animals.

The caudate nucleus, putamen and ventral portion of the mesencephalon from six normal rabbits were excised for fluorescence microscopy The caudate nucleus showed a strong green fluorescence, which was rather diffuse except at the margins, where it was seen to be confined to delicate, richly varicosed fibres. However in some optimal cases (when an unusually dry formaldehyde gas was used for the condensation reaction) such fibres appeared throughout the nucleus, although some diffusion artifacts were never entirely absent The fibres formed a dense network with abundant extremely small meshes, the only larger apertures being formed by non fluorescent cell bodies Few varicosed fibres also occurred, which were much coarser than the above-mentoined and had the same appearence as the noradrenaline-containing terminals in, *e g.*, the hypothalamus. A small number of similar fibres were also seen to run along arteries. The findings in the putamen were essentially the same as those in the caudate nucleus At the site equivalent to the substantia nigra in the ventral mesencephalon an accumulation of multipolar cell bodies displayed a green fluorescence of moderate intensity Their processes could be traced only for a short distance

In three groups of normal rabbits, each group consisting of three animals, the mesencephalon was divided through the aqueduct into one

dorsal and one ventral part. Fluorimetric determinations showed that almost all the DA occurred ventrally to the aqueduct. The mean values for DA content in the dorsal and ventral parts were 0 03 and 0.27 μg/g, respectively No significant amounts of DA could be detected in the brain parts caudal to the mesencephalon. On the other hand, NA showed a fairly equal distribution in the ventral and dorsal mesencephalon and in the pons.

Histochemical investigations of the corpus striatum in operated pigeons and rabbits are in progress a report on them will be submitted for publication separately

Discussion

From the results of our investigation it seems that the rabbit lesions in the ventral part of the mesencephalon after seven days caused a considerable decrease in DA concentration in the homolateral but not in the contralateral telencephalon and diencephalon. This decrease must be due to a loss of striata DA, since almost all of the DA in these brain parts is located in the corpus striatum. These observations, and the fact that the dorsal mesencephalon and the brain part caudal to the mesencephalon contain no significant amounts of DA, indicate that at least part of the striatal DA is localized in neurons originating from the ventral mesencephalon. Although the dopaminergic perikarya so far have not been localized, it seems likely that they may be found in the substantia nigra. In the rabbit this nucleus was found to contain nerve cell bodies displaying on histochemical examinations a fluorescence characteristic of primary catecholamines further this nucleus is the only mesencephalic structure containing appreciable quantities of DA (but no or extremely small amounts of NA) in the human brain (BERTLER 1960). Histochemically the caudate nucleus – as well as the putamen – of the normal rabbit was found to contain non-fluorescent nerve-cell bodies and a large amount of extremely fine and richly variosed fibres, displaying a high fluorescence typical of primary catecholamines. This seems to correspond well with the high amount of DA found in this nucleus, which contains only small amounts of NA (BERTLER & ROSENGREN 1959).

The assumption that dopaminergic neurons originate in the ventral mesencephalon and terminate in the corpus striatum of the mammalian brain finds strong support from the experiments on the pigeon brain. In this animal the paleostriatum augmentatum and neostriatum had histochemically the same appearance as the rabbit caudate nucleus and putamen, the only difference being that the fine fibres were even better observable. This finding is consistent with the view (JOHNSTON 1923 KAPPERS *et al.* 1960) that these two structures in the mammalian and avian brain

Table 3

Effect of unilateral lesions in the ventral mesencephalon of the rabbit on the catecholamine content of cerebral hemisphere plus the diencephalon.

Exp. nr	NA, µg/g		DA µg/g	
	op side	unop. side	op side	unop. side
1	0.13	0 38	0.31	0.57
2	0 10	0.23	0 42	0.45
3	0.20	0.24	0.20	0.44
4	0.08	0.15	0.48	0.4
5	0 06	0.12	0 45	0.54
6	0.09	0.11	0.35	0.48
7	0 13	0.27	0.59	0.64
8	0.17	0.27	0.59	0.86
9	0.08	0.20	0.05	0.37
10	0.15	0.18	0.37	0.45
11	0 14	0.21	0.51	0.54
Mean values	0.1	0.21	0.37	0.52

centration was considerably lower on the operated than on the unoperated side The NA values on the unoperated side were also significantly lower than those for untreated animals (table 3) The DA values obtained in the part frontal to the lesion were significantly lower than those obtained from the corresponding part of the unoperated side (table 3) which did not differ from those found in untreated animals.

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In three groups of normal rabbits, each group consisting of three animals, the mesencephalon was divided through the aqueduct into one

fibres in the brain parts frontal to the lesion derive from or pass through the mesencephalon. The pathway seems to be partly crossed, as the NA concentration decreased on both the operated and the unoperated side. The nuclei from which these fibres derive have not been identified.

Preliminary results from histochemical examinations show that the fibres to the hypothalamus are crossed, but that there is a less crossing over of the fibres going to the rich network of adrenergic fibres that has been found in the limbic system.

Summary

The striatal dopamine in the rabbit brain is localized in axons at least partly originating from the ventral mesencephalon. A corresponding adrenergic pathway has been directly demonstrated in the pigeon brain.

A large portion of the NA in the cerebral hemispheres and the diencephalon of the rabbit has been found to be derived from neurons coming from or passing through the mesencephalon.

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are homologous An unexpected finding was that the preterminal parts of the striatal terminals in the pigeon also developed a specific fluorescence. In the normal mammalian brain the content of adrenergic transmitter in the preterminal part of the neurons appears too low to be histochemically demonstrable Although the preterminal fluorescence was much weaker than the terminal fluorescence, it permitted the direct tracing of the adrenergic pathway back to a fluorescent cell group in the anterior mesencephalon Most of these cells were located ventrally however in contrast to the mammalian substantia nigra, this group also extended into the dorsal mesencephalon Although this directly demonstrates the mesencephalic origin of the striatal adrenergic neurons, it is not immediately evident which type of primary catecholamine is stored in these neurons. It is true that, in the areas where these fluorescent neurons arise and end, the highest concentrations of DA also occur and in fact DA occurs at a level making it improbable that the DA storing structure would not be histochemically demonstrable. Further the outcome of the lesion experiments supports the view that the striatal DA is stored in neurons which because of the observed distribution of DA in the pigeon brain (cf table I) must originate from the ventral mesencephalon. Thus, histochemical and chemical findings both indicate that the adrenergic connection between the ventral mesencephalon and the corpus striatum is at least in part dopaminergic. At present the possibility cannot be excluded that there also occur noradrenergic neurons in this system since NA is present in the dissected part of the brain, mainly consisting of the paleostriatum augmentatum and the neostriatum and also in large amounts in the ventral mesencephalon. It should be kept in mind however that the mesencephalic NA may be stored in the abundant system of highly fluorescent varicose terminals found in this area. Further the occurrence of NA in the dissected part of the corpus striatum may be due to the unavoidable contamination by tissue from adjacent areas The DA storing neuron is similar to the noradrenergic and 5-hydroxytryptaminergic peripheral and central neurons as well as to the adrenaline-containing neuron in the frog and the dopaminergic neuron in certain invertebrates (cf FALCK 1964) The intraneuronal distribution of amine is the same, most of it being accumulated in the terminal part morphologically it also consists of a smooth preterminal and a varicose terminal part. A remarkable difference is that the terminal of the DA neuron studied in this investigation is much finer and more richly varicosed than the noradrenergic terminals so far identified in brain

It was also found that the concentration of NA in the hemispheres plus the diencephalon decreased in the rabbit after production of lesions in the ventral mesencephalon. Thus at least part of the NA-containing

could not be shown. ALEXANDER & WEAVER (1955) showed that in mice amphetamine alone was without any effect on convulsions elicited by the maximal electroshock seizure procedure or by subcutaneous injection of pentetrazole. The ED50 of phenobarbital in the electroshock test was not altered when up to 28 / amphetamine was added to the phenobarbital dose. In the pentetrazole test the same combination appeared to elevate the effective dose of phenobarbital slightly but not significantly The relevance of these results may be questioned, since both drugs were given 3 hours before the electrical or chemical induction of the convulsion, i.e. at a time when the maximal effect of amphetamine on the mouse is already falling off.

The paucity of available experimental evidence prompted us to investigate the effect of combining the use of amphetamine with that of common anticonvulsant drugs in a model for grand mal epilepsy (maximal electroshock seizure) and one for petit mal epilepsy (pentetrazole seizure threshold test). Since there is good clinical agreement that amphetamine has no antagonistic effect on the anticonvulsant activity of the other medication, the experiments should also test the reliability and adequacy of the pharmacological tests mentioned for predicting clinical efficacy

Method

All experiments were done on mice of both sexes of the Leo strain, weighing 20-24 g.

The threshold for the extensor component of the electroshock procedure was determined by varying the intensity of the current. A current of 50 cycles/sec. and 0.2 sec. duration was applied by corneal electrodes, and each current intensity was tested in groups of 10–20 mice. Likewise the CD50 (dose provoking convulsions in 50 / of the animals) for pentetrazole was determined by intravenous injection of increasing doses to groups of 10 mice. A clonic convulsion with loss of body righting reflexes was taken as end-point.

The anticonvulsant activity was determined by the methods of SWINYARD *et al.* (1952)

1 Maximal electroshock seizure test with a current intensity of 50mA, taking the abolition of the tonic extensor component as end-point for anticonvulsant action

2 The pentetrazole seizure threshold test in which the mice received a subcutaneous injection of 100 mg/kg pentetrazole (CD95) The mice were considered protected if they did not show clonic convulsions with loss of the upright position within 30 min.

All median effective doses and currents were determined by the graphical method of LITCHFIELD & WILCOXON (1949). The differences in the effective doses between mice pretreated and not pretreated were tested for statistical significance by the methods described in the same publication

The anticonvulsant drugs tested in the maximal electroshock seizure test were phenobarbital and diphenylhydantoin in the pentetrazole seizure threshold test phenobarbital, trimethadione and ethosuccinimide were tested. The anticonvulsant doses of these drugs are given in terms of the free acids. The ED50 for each of these

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Note on the Interactions of Amphetamine with Anticonvulsant Drugs

By

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Since the end of the thirties amphetamine has often been used for treating the different forms of epilepsy In most instances the drug was given with the intention of counteracting the sedative side effect of anti convulsant medication produced by phenobarbital (phenemalum NFN) or less often by diphenylhydantoin (phenytoinum NFN) (COHEN & MYERSON 1938 COHEN *et al* 1940 ROBINSON 1941 COOK & DOLE 1942 KRÖBER 1949) According to a recent review by SCHOLL (1962) the combined use of amphetamine is possible with nearly all current anticonvulsant drugs used. All authors agree that amphetamine has no adverse effect on the antiepileptic action of other medication some of them even hold that there is an additive effect of amphetamine in suppressing the number of *grand mal* seizures, while such an effect is denied by STONE *et al* (1940). In *petit mal* epilepsy there is some evidence for a positive effect caused by amphetamine alone on the number of spells (STRAUSS 1944 LIVINGSTON *et al* 1948) Since petit mal spells occur most often during periods of mental inactivity and are only rarely seen during periods of alertness, such an effect of amphetamine could easily be understood (GOODMAN *et al* 1949)

In spite of fairly frequent references to the use of amphetamine for treating epilepsy there are few experimental studies of the effect of amphetamine alone, or along with classical antiepileptic medication, on anticonvulsant activity

TAINTER *et al* (1943) noted a weak elevation of the threshold for electrical convulsions in rabbits after oral application of DL or D-amphetamine, but these results are not very convincing, since a dose-effect relationship

could not be shown. ALEXANDER & WEAVER (1955) showed that in mice amphetamine alone was without any effect on convulsions elicited by the maximal electroshock seizure procedure or by subcutaneous injection of pentetrazole. The ED50 of phenobarbital in the electroshock test was not altered when up to 28 / amphetamine was added to the phenobarbital dose. In the pentetrazole test the same combination appeared to elevate the effective dose of phenobarbital slightly but not significantly. The relevance of these results may be questioned, since both drugs were given 3 hours before the electrical or chemical induction of the convulsion, i.e. at a time when the maximal effect of amphetamine on the mouse is already falling off.

The paucity of available experimental evidence prompted us to investigate the effect of combining the use of amphetamine with that of common anticonvulsant drugs in a model for grand mal epilepsy (maximal electroshock seizure) and one for petit mal epilepsy (pentetrazole seizure threshold test). Since there is good clinical agreement that amphetamine has no antagonistic effect on the anticonvulsant activity of the other medication, the experiments should also test the reliability and adequacy of the pharmacological tests mentioned for predicting clinical efficacy.

Method

All experiments were done on mice of both sexes of the Leo strain, weighing 20–24 g.

The threshold for the extensor component of the electroshock procedure was determined by varying the intensity of the current. A current of 50 cycles/sec. and 0.2 sec. duration was applied by corneal electrodes, and each current intensity was tested in groups of 10–20 mice. Likewise the CD50 (dose provoking convulsions in 50 / of the animals) for pentetrazole was determined by intravenous injection of increasing doses to groups of 10 mice. A clonic convulsion with loss of body righting reflexes was taken as end-point.

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1. Maximal electroshock seizure test with a current intensity of 50mA, taking the abolition of the tonic extensor component as end-point for "anticonvulsant action.
2. The pentetrazole *seizure threshold* test in which the mice received a subcutaneous injection of 100 mg/kg pentetrazole (CD95). The mice were considered protected if they did not show clonic convulsions with loss of the *upright position within* 30 *min.*

All median effective doses and currents were determined by the graphical method of LICHTFIELD & WILCOXON (1949). The differences in the effective doses between mice pretreated and not pretreated were tested for statistical significance by the methods described in the same publication.

The anticonvulsant drugs tested in the maximal electroshock seizure test were phenobarbital and diphenylhydantoin in the pentetrazole seizure threshold test phenobarbital, trimethadione and ethosuccinimide were tested. The anticonvulsant doses of these drugs are given in terms of the free acids. The ED50 for each of these

compounds was determined alone and along with doses of 3 5 or 8 mg/kg DL-amphetamine sulphate. All compounds were applied orally the whole volume applied did not exceed 10 ml/kg, in order to avoid unspecific effects of the water load (KAMMANN & FREY 1963). The time of application was so chosen that the drugs displayed the maximum activity when the convulsions were elicited, that is 2 hours before the application of electroshock or pentetrazole for phenobarbital and diphenylhydantoin and 1 hour before for trimethadione, ethosuccinimide and amphetamine.

Results

The results are summarized in tables 1–3 From table 1 it can be seen that oral doses of 3 5 and 8 mg/kg DL amphetamine sulphate (corresponding to 1 1 1 8 and 2.9 mg/kg of the centrally active D-form of the base) are without any influence on the convulsion threshold in otherwise untreated mice. The threshold current in the electroshock procedure was only elevated significantly by the highest dose of amphetamine. Since we confirm the results of MEYER & MEYER BURG (1964) that the threshold current in this test may show some diurnal variations, this result should be considered as being at the edge of significance

In the maximal electrochock seizure test (table 2) amphetamine proved to be without any effect on the anticonvulsant activity of phenobarbital. The ED50 for diphenylhydantoin was elevated by the two lower doses of amphetamine, but with the highest dose of amphetamine this effect was less marked and indeed no longer significant

In the pentetrazole seizure threshold test (table 3) the ED50 for phenobarbital was only slightly elevated by the two lower doses of amphetamine however with the highest dose of the amine the elevation became significant at the 5 % level The elevation of the anticonvulsant dose of phenobarbital was paralleled by a progressive flattening of the dose response

Table 1

Effect of oral pretreatment with DL amphetamine on convulsive thresholds (CD50) for the extensor component of the electroshock seizure and for the clonic convulsion after intravenous injection of pentetrazole in mice.

Procedure	Convulsive threshold (CD50)			
	without amphetamine	after pretreatment with amphetamine[1]) 3 mg/kg	5 mg/kg	8 mg/kg
Electroshock	20.5 mA (19 5–21 5)	2[illegible] mA (21 24)	2[illegible] mA ([illegible]0–[illegible]4)	[illegible]5 mA (24–26) $p < .05$
Pentetrazole	28 mg/kg ([illegible]5–31)	29 mg/kg (26–33)	29 mg/kg (26–31)	[illegible]6 mg/kg (23 [illegible]9)

1) Amphetamine was given orally 1 hour before application of electroshock or injection of pentetrazole.

Table 2

Effect of oral pretreatment with DL amphetamine on anticonvulsant ED50 of phenobarbital and diphenylhydantoin in the maximal electroshock seizure test. Phenobarbital and diphenylhydantoin were given orally 2 hours, amphetamine 1 hour before application of the electroshock.

Anticonvulsant	ED50 (mg/kg)			
	without amphetamine	after pretreatment with amphetamine		
		3 mg/kg	5 mg/kg	8 mg/kg
Phenobarbital	13(12 15)	14(12–16)	15(13–18)	11(9.3–13)
Diphenylhydantoin	14(12–16)	19(15.5–24) $p < .05$	19(15.5–24) $p < .05$	17.5(12.5–24)

Table 3

Effect of oral pretreatment with DL-amphetamine on anticonvulsant ED50 of phenobarbital, trimethadione and ethosuccinimide in the pentetrazole seizure threshold test. All compounds were applied orally phenobarbital 2 hours, the other ones 1 hour before subcutaneous injection of pentetrazole.

Anticonvulsant	ED50 (mg/kg)			
	without amphetamine	after pretreatment with amphetamine		
		3 mg/kg	5 mg/kg	8 mg/kg
Phenobarbital	14(11–17)	16.5(12–22)	18(13–24)	22(17 27) $p < .05$
Trimethadione	310(240–400)	460(350–610) $p < .05$	540(390–750) $p < .05$	700(540–900) $p < .01$
Ethosuccinimide	190(160–230)	300(260–360) $p < .02$	300(260–360) $p < .02$	360(300–420) $p < .01$

curves the value for S rose from 1 51 for phenobarbital alone to 1 81 for its action with 8 mg/kg amphetamine.

Contrary to the results with phenobarbital, amphetamine strongly counteracted the anticonvulsant effects of trimethadione and ethosuccinimide. With these two drugs the ED50 was already elevated significantly by simultaneous application of 3 mg/kg amphetamine and given with 8 mg/kg amphetamine the doses of the two anticonvulsants had to be about doubled in order to afford protection against the convulsive action of pentetrazole.

It should be mentioned that the centrally stimulating effect of amphetamine could be observed with all amphetamine doses used and in all combinations with anticonvulsant drugs.

Discussion

The investigation described here into the role of amphetamine in experimental epilepsy has revealed first, that amphetamine has almost no effect on the thresholds for electro- and chemo-seizures second that it does not antagonize the effect of phenobarbital and diphenylhydantoin in the maximal electroshock seizure test to any reasonable degree and third that in the pentetrazole seizure threshold test it shows strong antagonism to the anticonvulsant action of trimethadione and ethosuccimide but only a weak effect against that of phenobarbital Of these 3 findings the first two are in agreement with empirical clinical knowledge, the third – which might be regarded as the most likely for a central stimulant – is not. For phenobarbital our results confirm earlier experimental findings by ALEXANDER & WEAVER (1955)

At the first glance one might expect antagonism between amphetamine, known to have a stimulant action both in the cortex and in the brain stem, and anticonvulsant drugs, generally regarded as central depressants. But besides a facilitatory action of amphetamine on central and peripheral synapses there is some evidence that the drug at the level of the reticular formation may have an inhibitory action on synaptical transmission already facilitated or activate inhibitory mechanisms (XAVIER & TIMO-IARIA 1964 LUCO et al 1949) The remaining gap between our rather low oral doses and the relatively high doses applied intravenously intra-arterially or direct into the reticular formation by the authors named may be explained by the fact that the last mentioned mainly worked on anaesthetized animals.

The effect of amphetamine at the level of the reticular formation reminds one of the ability of the drug to liberate noradrenaline from the brain (MCLEAN & MCCARTNEY 1961 HIGUCHI et al 1962) However at our present state of knowledge, it is difficult to see any relation between this effect and the action of anticonvulsants. According to the work of MCLEAN & MCCARTNEY on the rat it is unlikely that the release of noradrenaline is fairly pronounced so soon as one hour after the oral application of relatively low doses of amphetamine

Thus, we have at present no satisfactory explanation for the nearly absolute lack of its effect on the convulsive threshold for electrical current and intravenous pentetrazole The highest dose of amphetamine raised the threshold for the maximal electroshock just significantly an effect that could be considered to be in agreement with the mentioned results of XAVIER & TIMO-IARIA on the cat and TAINTER et al (1943) on the rabbit, as well as with the electroencephalographic observations of WEINLAND &

KRÖBER (1950) reporting on some reduction in the seizure potentials after administration of methamphetamine to man.

However taking into account the potential elevation of the threshold for electroconvulsions, amphetamine should have some potentiating action on drugs active in the maximal electroshock seizure test. Our results show that this is not so with phenobarbital with diphenylhydantoin there was a slight but significant antagonistic effect from doses of 3 and 5 mg/kg but not from 8 mg/kg DL amphetamine.

Summarizing, it seems justifiable to conclude that a combination of *amphetamine with drugs of reasonable* activity in the maximal electroshock seizure test does not have any notable influence on their anticonvulsant efficacy This is again in good agreement with clinical reports on such combinations for the treatment of grand mal epilepsy which all lack conclusive evidence for any positive or negative effect of amphetamine on the rate and severity of seizures.

Though amphetamine pre-treatment proved to be without effect on the convulsive threshold for intravenously injected pentetrazole, a clear antagonism between amphetamine and the anticonvulsant action of drugs in the pentetrazole seizure threshold test appeared. This antagonism was relatively weak with phenobarbital, with which it first became significant at the highest dose level. An explanation for this difference from the results with trimethadione and ethosuccinimide could lie in the known weak barbiturate-antagonistic effect of the amphetamines (FREY & KRAUSE 1957). More difficult is an explanation for the powerful antagonism of amphetamine to trimethadione and ethosuccinimide, both known to be fairly specific antagonists of pentetrazole. That this effect of amphetamine has no relation to pentetrazole is suggested by experiments summarized in table 4, in which the weak and *unspecific* anticonvulsant effect of trimethadione in the maximal electroshock seizure test is seen to be antagonized as well. One may imagine a competition between amphetamine and these anticonvul-

Table 4

Effect of oral pretreatment with 3 mg/kg DL-amphetamine on anticonvulsant effect of trimethadione in the maximal electroshock seizure test. Each compounds was given orally 1 hour before the electroshock.

Treatment	ED50 of trimethadione g/kg	
Trimethadione	0 87(0.79–0 96)	
Trimethadione + 3 mg/kg amphetamine	1 34(1 15–1.55)	$p < .02$

sants for sites, at present unknown, where the latter display their anticonvulsant effect. Amphetamine should here have a higher affinity than the anticonvulsant for these sites and at the same time be devoid of any agonistic or antagonistic activity which would have resulted in an effect on the pentetrazole seizure threshold. Another possibility would be a potentiating action of amphetamine on the central stimulant properties of the anticonvulsants unrelated to pentetrazole. In unpublished results with ethosuccinimide, such stimulant effects could be demonstrated. A oral dose of 50 mg/kg in the mouse caused a significant prolongation of hexobarbital sleeping time, but this prolongation declined with higher doses until after pretreatment with 400 mg/kg, there was a trend to shortening of the sleeping time. After intravenous injection of lethal amounts of ethosuccinimide death occurred with clonic and tonic convulsions resembling those after pentetrazole only in the surviving animals was this phase succeeded by a period of central depression (sleep). Likewise, with toxic doses of trimethadione symptoms of central excitement predominated in the mouse.

On the other hand our results in the chemoseizure test are not consistent with clinical experience in the treatment of petit mal epilepsy Amphetamine is given to the patient in order to counteract sedative side-effects of trimethadione as well as of ethosuccinimide (GOODMAN & GILMAN 1958 SCHOLL 1962) and no adverse effects of the combination are *mentioned* The possibility that the activity of amphetamine alone against petit mal spells (STRAUSS 1944 LIVINGSTON *et al* 1948 GOODMAN *et al* 1949) could just outweigh an antagonism to the anticonvulsant drugs in question seems too hypothetical to be accepted

Thus our results must be taken as evidence that the pentetrazole seizure threshold test is no very good model for petit mal epilepsy in man. Our results with this test thus seem to be of merely theoretical interest and have presumably no bearing on clinical therapy On the other hand, the good parallelism between the results in maximal electroshock seizures and clinical treatment of grand mal epilepsy once more demonstrates the adequacy of this pharmacological model

Summary

The widely used combination of amphetamine with nearly all anticonvulsant drugs in the treatment of human epilepsy leads to a study of the interactions between the two medications in tests pharmacologically employed for the measurement of anticonvulsant activity in mice. In the maximal electroshock seizure test amphetamine proved to have no clear effect on the anticonvulsant activity of phenobarbital and diphenyl-

hydantoin. The results are thus in good agreement with clinical experience in grand mal epilepsy. However in the pentetrazole seizure threshold test, usually taken as model for petit mal epilepsy amphetamine showed a weak antagonism to the anticonvulsant action of phenobarbital and a powerful one to that of trimethadione and ethosuccinimide. These results are at variance with clinical experience in the treatment of petit mal, thus showing the inadequacy of this pharmacological model.

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Summary

The widely used combination of amphetamine with nearly all anticonvulsant drugs in the treatment of human epilepsy leads to a study of the interactions between the two medications in tests pharmacologically employed for the measurement of anticonvulsant activity in mice. In the maximal electroshock seizure test amphetamine proved to have no clear effect on the anticonvulsant activity of phenobarbital and diphenyl

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Paper Chromatographic Determination of Dicoumarol in Biological Materials

By

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A quantitative method for determining dicoumarol on paper chromatograms has recently been described (CHRISTENSEN 1964 a). This method has been further developed to make possible determinations of dicoumarol in biological materials. In this paper we have described the extended method, with which we have compared ultraviolet spectrophotometric methods for determining dicoumarol

Experimental methods

Principles

Dicoumarol is extracted from biological materials by an organic solvent from which it is re-extracted with dilute sodium hydroxide. After acidification it is extracted into chloroform, which is evaporated. The residue is dissolved in a small volume of chloroform and submitted to paper chromatography This procedure has been described in a previous paper (CHRISTENSEN 1964 a)

General procedure in detail. Place 4 ml of the biological material (*e g* plasma, urine or tissue homogenate) in a 100 ml glass-stoppered flask. Homogenates should not contain more than 35 g of tissue in a total of 100 ml. Add 0.5 ml of 2 M-$(NH_4)_2SO_4$, adjust pH to 4–5 by means of 0.1 or 1 0 M HCl, and bring the volume to 5 ml with water

Add 25 ml of ethylene chloride (Merck, extra pure) to the flask, and shake mechanically for one hour at room temperature. Transfer to a centrifuge tube and centrifuge. Aspirate the two upper layers, consisting of water phase and precipitated protein. Transfer 20 ml of the organic phase to a 100 ml glass-stoppered flask, add 4 ml of 2.5 M-NaOH and shake mechanically for 10 min. Transfer to a centrifuge tube and

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Because a purer extract is obtained by this modification, a quantitative spectrophotometric assay can be carried out on the aqueous sodium hydroxide solutions by which the heptane is extracted. Up to this point the method is essentially the same as the method of AXELROD *et al.* (1949).

Recovery experiments were performed on different biological materials (plasma, urine and homogenates) to which the drug had been added.

Total recoveries of dicoumarol injected intravenously into 2 rats were also determined. Each rat received 5.2 mg of the drug dissolved in 0.1 M-NaOH, the solution (0.5 ml) being slowly injected into the tail vein. Five minutes later the rats were killed under ether anaesthesia by heart puncture, and as much of their blood was collected as possible. The rats were skinned, the gastro-intestinal tracts removed, and the carcases minced in a meat mincer. The skin, after depilation, was frozen in a bath of dry ice and absolute ethanol and then crushed in a mortar. The skin, the carcass and the gastro-intestinal tract were next homogenised separately in 0.9 % (w/v) sodium chloride by means of an Ultra-Turrax homogenisator. The homogenates and the blood were then analysed by the paper chromatographic method.

Comparative determinations of dicoumarol by paper chromatography and ultraviolet spectrophotometry. Four samples of human plasma, obtained from patients receiving anti-coagulant theraphy with dicoumarol, were analysed for their content of dicoumarol by paper chromatography and by the ultraviolet spectrophotometric method of AXELROD *et al.* (1949).

Likewise, four livers, obtained from rats to which dicoumarol had been administered by intravenous injection some hours before, were analysed by paper chromatography and by the ultraviolet spectrophotometric method of WEINER *et al.* (1950).

Results

The amount of dicoumarol that could be extracted from tissues by a single extraction depended on, among other factors, the organic solvent used and the pH to which the biological material had been adjusted before the extraction. Among several organic solvents tested at different pH values ethylene chloride was found most satisfactory especially at pH 4 to 5 (table 1). However with human plasma high recovery rates were

Table 1

Extraction of dicoumarol from biological materials with ethylene chloride. Recovery of dicoumarol is shown in relation to the pH of the biological material at the time of extraction. Determinations were performed by paper chromatography.
Number of experiments in parenthesis.

pH of water phase	Mean recovery per cent	
	Muscle	Liver
< 2	67 (3)	80 (9)
4–5	95 (5)	98 (5)
6–7	92 (6)	94 (6)
7–8	85 (6)	

centrifuge Pipette 3 ml of the sodium hydroxide into a 25 ml glass-stoppered test tube. (A preliminary check of the amount of dicoumarol in the sample may be achieved at this point by determining the absorption of the sodium hydroxide solution at 314 mµ). Next, place the tube in a water ice bath for 5 min. add conc. HCl (1 ml) and 8 ml of chloroform (Merck, pro analysi). Shake the tube mechanically for 15 min. and allow the two phases to separate completely Aspirate the water phase carefully and pipette 7 ml of the chloroform into a cylindrical glass vessel measuring 2.5 cm in diameter and 5 cm in height. Evaporate the extract to dryness, eventually using a warm air stream.

Dissolve the residue in chloroform (*e g* 1 ml) and apply a known fraction (*e.g* 750 µl) to the chromatogram by means of the applicator mentioned below

Application of the sample to the paper chromatogram Because of the low solubility of dicoumarol in organic solvents, it was necessary to apply as much as 750 µl of chloroform to the paper A simple and inexpensive device has been developed for applying such quantities of chloroform to chromatograms.

The applicator is prepared from a glass tube (outer diameter about 8 mm, wall thickness about 2 mm) which is drawn to a capillary at one end, the other end remaining open. The broad end of the tube should be made 3–4 cm in length and the capillary 12–16 cm. The applicator is by means of a clamp fixed in a vertical position, the end of the capillary being in contact with the paper at the point where the spot is to be applied. It is important that the tip of the capillary be also fixed in its position, for example by means of a small wire loop

A measured volume of the sample is placed in the larger part of the applicator which serves as a reservoir By capillary force fluid runs from the reservoir on to the paper forming a spot, whose evaporation rate is increased considerably by means of a positive air stream

By trial and error the thickness of the capillary is made such as to permit delivery of a spot of appropriate size. If the fluid runs too slowly a rubber stopper may be forced into the open end of the applicator to give an increased pressure.

After the sample has been applied to the paper the residue from the applicator is removed by washing twice with chloroform (25 µl) and collected on the same spot. The unknown samples, together with two standards of dicoumarol, should be run on the same paper

The chromatographic procedure and the quantitative determinations The chromatogram is developed overnight by one-dimensional technique with n-butanol 3 M aqueous ammonia, 1 1 (v/v)

After drying, the chromatogram is sprayed with diazotized sulphanilic acid and then dried again, the spots containing dicoumarol are eluted, and the optical densities of the eluates are determined at 415 mµ, all as described in the previous paper (CHRISTENSEN 1964 a). Standards of dicoumarol that have been put through the whole procedure, as well as blank samples obtained from suitable places on the chromatogram, are also measured.

The optical density of the unknown (OD_{A}) and the mean values of the optical densities of the standards (OD_{ST}) and the blanks (OD_{BL}) are used for calculating the amount of dicoumarol in the unknown sample by means of the equation

$$\mu g \text{ dicoumarol in the sample} = \frac{OD \quad - OD}{OD_{ST} - OD_{BL}} \times (\mu g \text{ dicoumarol in standard})$$

Modified method for human plasma For determining dicoumarol in human plasma a slight modification is recommended. The extraction with ethylene chloride at pH 4–5 is replaced by one with n-heptane at pH 1–2 otherwise the procedure is the same.

Table 3

Recovery of dicoumarol from two rats. The animals each received 5.20 mg of the drug intravenously 5 min. before they were killed. Blood, skin, gastro-intestinal tract and carcass were separately analysed for their content of dicoumarol by paper chromatography. The weights of the animals 1 and 2 were 169 and 195 g respectively
Gast.-intest. = gastro-intestinal tract.

Animal No.	Recovery mg				Total recovery	
	Blood	Skin	Gast. intest.	Carcass	mg	per cent
1	0.99	0.51	0.33	3.37	5.20	100
2.	0.83	0.38	0.26	3.91	5.38	103

Table 4

Comparison between dicoumarol determinations performed by paper chromatography and ultraviolet spectrophotometry (AXELROD *et al.* 1949). Plasma samples were obtained from patients receiving anti-coagulant therapy with dicoumarol. Concentrations in µg/ml.

Plasma sample No.	Paper chromatography	Ultraviolet spectrophotometry
1	7.6	8.1
2	17.4	17.3
3	27.5	26.5
4	16.8	17.4

Table 5

Comparison between dicoumarol determinations performed by paper chromatography and ultraviolet spectrophotometry (WEINER *et al.* 1950). Rat livers were obtained from animals injected intravenously with dicoumarol. Concentrations in µg/g of wet tissue weight.

Rat liver No	Paper chromatography	Ultraviolet spectrophotometry
1	38.0	49.4
2	34.2	51.0
3	33.2	31.2
4	19.4	18.5

Table 2

Recovery of dicoumarol added to various biological materials and determined by paper chromatography The drug (50 µg) was added to each sample, consisting of 4 ml of urine, 1 ml of *plasma or 4 ml of homogenate* (containing 0 8 g of tissue) The determinations of dicoumarol were based on aqueous standards of the drug carried through the whole procedure, as described in the text.

Experiment No	Recovery rate per cent			
	Urine	Plasma	Muscle tissue	Liver tissue
1	102	102	82	106
2	102	103	102	97
3	100	104	101	99
4	98	104	97	97
5	98	104	95	92
m	100 0	103 4	95 4	98.2
s	±2.0	±0.9	±8.0	±5 1

also obtained if the extraction was performed at pH 1–2 with n heptane as the solvent.

By the procedure described the drug is extracted from one phase into another in 3 stages From the measured portions of these extracts used it can be calculated that 52 5 / of the dicoumarol in the original sample should be expected in the final chloroform extract Usually three quarters of this amount is applied to the paper chromatogram, thus reducing the percentage to 39 4 Analysing watery standards of dicoumarol on diffe rent occassions gave a mean value of 37 1 / (n = 14) thus 94 3 / of the amount of dicoumarol expected was recovered on the final chroma togram. Further a certain day to-day variation in this recovery rate was recorded These systematic errors of the method are, however removed by the use of dicoumarol standards put through the whole procedure.

From table 2 it appears that the recovery rate obtained with dicoumarol added to various biological materials was nearly 100%, but that the recoveries depended somewhat on the materials examined.

Table 3 shows the total recoveries of dicoumarol administered intra venously into two rats The results indicate almost complete recovery In these experiments the chromatograms of the gastro-intestinal tracts revealed a substance not completely separated from dicoumarol

Tables 4 and 5 show the comparative determinations of dicoumarol by the paper chromatographic and ultraviolet spectrophotometric methods The results obtained with human plasma (table 4) were similar and the paper chromatograms did not reveal spots other than those of dicoumarol For rat livers (table 5) the results differed rather markedly in two experi

indicate that both of these methods determine the drug accurately. Interference by metabolites presumably does not take place, as was also found by AXELROD *et al.* (1949), who were unable to demonstrate such interference when assaying dicoumarol in human plasma by their ultraviolet spectrophotometric method. This fairly simple method is thus in most instances satisfactory for determining dicoumarol in human plasma, but paper chromatography may help to give further information about that material that, in the ultraviolet spectrophotometric assay, absorbs light at 314 mμ.

For the assay of dicoumarol in rat livers, the rather divergent results between those obtained by ultraviolet spectrophotometry and paper chromatography can be explained by the presence of metabolites interfering with the assay of the drug by the first-mentioned method. This explanation is supported by the presence, in the livers of dicoumarol-treated rats, of the metabolite, B-055, which strongly absorbs light at 314 mμ if dissolved in aqueous sodium hydroxide, as in the ultraviolet spectrophotometric assay of dicoumarol. Incomplete extraction of dicoumarol from the tissues at a strongly acid pH may also contribute to the divergent results obtained. Paper chromatographic assay of dicoumarol in tissues therefore seems preferable to ultraviolet spectrohpotometry at least for certain purposes, and may be useful in studying the distribution and metabolic fate of the drug in animals. The method is however more complicated and laborious than the ultraviolet spectrophotometric method.

Several principles, of which paper chromatography is the most important, have been used in our method to separate dicoumarol from related substances. As shown in a previous paper (CHRISTENSEN 1964 a) separation of several 4-hydroxycoumarin derivatives may be obtained by paper chromatography, and the present study indicates that dicoumarol also can be separated from its metabolites in this way. These facts, and the failure to demonstrate any spots on chromatograms of tissues from untreated rats, indicate the specificity of the method described.

Summary

A method is described for determining dicoumarol in biological materials by means of paper chromatography. The mean recovery rates observed with dicoumarol added to urine, plasma, muscle, and liver tissues were 100, 103, 95 and 98 % respectively. The lower limit of the method is about 6 μg of dicoumarol and the accuracy is estimated to be ±3 to ±10 per cent depending on the material to be analysed. The new method has been compared with ultraviolet spectrophotometric methods for assaying dicoumarol in plasma and other biological materials.

ments (no 1 and 2) and the paper chromatograms revealed the presence in the livers of dicoumarol treated rats of several unknown substances. One of these substances, with an R_f-value of 0 55 was always the prevailing one and was especially abundant present in the rat livers from experiments 1 and 2. It reacted with diazotized sulphanilic acid to give a coloured dye-stuff indistinguishable from that obtained with dicoumarol. As all of the unknown substances mentioned were apparently not present in the livers of rats untreated with the drug, they were presumed to be metabolites of dicoumarol

Discussion

According to most of the methods hitherto described, dicoumarol is extracted at an acid reaction from the biological material before its assay and it is therefore of importance for the drug to be extracted at a reproducible rate With human plasma, to which most methods have been devoted high extraction rates have generally been reported Thus, a mean recovery rate of 97 9 / of dicoumarol added to human plasma was obtained by the ultraviolet spectrophotometric method of AXELROD *et al* (CHRISTENSEN 1964 b) However as far as is known few figures are available for recovery of dicoumarol from other biological materials. With the ultraviolet spectrophotometric method of WEINER which is the method commonly used for tissue determinations, GREEN *et al* (1956) reported recoveries from 84 to 103 / of dicoumarol added to liver homogenates. We have also used this method in a series of recovery experiments and obtained recovery rates of 85–103 / with kidney homogenates and 78–85 / with muscle tissue homogenates

As indicated by these results, the recovery rates are somewhat variable if dicoumarol is extracted from tissues at a strongly acid pH our study indicates that an almost complete recovery of the drug can be achieved by extraction at pH 4–5

The accuracy of assaying dicoumarol by paper chromatography depends, among other things, on the material to be analysed In an experiment with human plasma containing on the average 16 9 µg/ml of dicoumarol the accuracy (as the standard deviation) was found to be ± 2.2 / (n = 8) With other materials it was somewhat less.

The lower limit for obtaining a reasonably accurate estimate of dicoumarol by paper chromatography is of the magnitude 5–7 µg in the original sample, but amounts as little as 1–2 µg can be detected qualitatively

The nearly identical results obtained by assaying dicoumarol in human plasma by ultraviolet spectrophotometry or by paper chromatography

indicate that both of these methods determine the drug accurately Interference by metabolites presumably does not take place, as was also found by AXELROD *et al.* (1949), who were unable to demonstrate such interference when assaying dicoumarol in human plasma by their ultraviolet spectrophotometric method. This fairly simple method is thus in most instances satisfactory for determining dicoumarol in human plasma, but paper chromatography may help to give further information about that material that, in the ultraviolet spectrophotometric assay absorbs light at 314 mμ.

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Summary

A method is described for determining dicoumarol in biological materials by means of paper chromatography The mean recovery rates observed with dicoumarol added to urine, plasma, muscle, and liver tissues were 100, 103 95 and 98 / respectively The lower limit of the method is about 6 μg of dicoumarol and the accuracy is estimated to be ±3 to ±10 per cent depending on the material to be analysed. The new method has been compared with ultraviolet spectrophotometric methods for assaying dicoumarol in plasma and other biological materials.

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Studies on the Fate of Intravenous Dicoumarol in the Rat

By

Flemming Christensen

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The distribution and elimination of dicoumarol in animals have already been studied by several investigators (e g WEINER *et al.* 1950 LEE *et al* 1950 HAUSNER *et al.* 1951 GREEN *et al* 1956). The spectrophotometric methods and the tracer methods, as used for determining dicoumarol in these investigations, are not entirely specific for the drug and metabolites may interfere with its determination. A new paper chromatographic method for determining dicoumarol (CHRISTENSEN 1964) is apparently more specific than those mentioned above, and a study of the distribution and elimination of dicoumarol in rats has been made by this method.

Experimental methods

White female rats weighing about 190 g were fed on a commercial chicken diet. Dicoumarol (5.2 mg) dissolved in 0.1 M-NaOH was slowly injected into the tail vein of each rat at the beginning of the experiment. The solution contained 10 mg of dicoumarol per ml and was prepared immediately before use. The rats were then placed, with free access to water and food, in metabolic cages for the collection of urine and faeces. At various times (one to forty-eight hours) after the injection of dicoumarol the animals were killed by heart puncture under ether anaesthesia, and as much blood as possible was withdrawn to reduce the amount of blood remaining in the tissues. The organs to be analysed and the entire gastro-intestinal tract, with its contents, were removed and weighed. No attempt was made to separate the gastro-intestinal tract proper from its content. Each rat was then skinned, and the skin, after depilation and weighing, was frozen in a bath of dry ice with absolute ethanol and crushed in a mortar the carcass was minced in a meat mincer The organs and tissues thus treated were then homogenized with 0.9 % (w/v) sodium chloride in an Ultra-Turrax homogenizer The homogenates as a rule contained 25–35 % (w/v) of tissue. Homogenates of faeces were also prepared.

The homogenates, plasma and urine were then analysed by quantitative paper chromatography as described by CHRISTENSEN (1964) The chromatograms were developed by n-butanol 3 M aqueous ammonia (1 1 v/v) The determinations were performed in duplicate when sufficient material was available (e g plasma, liver carcass and gastro- intestinal tract).

In 2 rats, killed 5 min. after the injection of dicoumarol only the total amount of dicoumarol remaining in the organisms at this time was determined. This was done by analysing separately the skin, gastro-intestinal tract, blood, and carcass.

Results

Tissue distribution of dicoumarol in rats after intravenous administration of the drug is given in table 1 The plasma level of the drug decreased in accordance with those of the different tissues examined. Among these, liver tissue showed the highest level of dicoumarol, and it is noted that the ratio of dicoumarol weight in the liver to that in plasma tended to increase at 36 and 48 hours The concentrations of dicoumarol in certain tissues (*e g* brain, muscle, spleen) were always low and erythrocytes (no values reported in the table) contained only traces of dicoumarol even at high plasma concentrations.

Rate of disappearance of dicoumarol from the whole rat was estimated from the decline in the total amount of dicoumarol that could be recovered from the individual animals at various times after intravenous administration of the drug. The total amount of dicoumarol in a rat, calculated from the amounts present in the tissues was expressed as a percentage of the dose injected and plotted logarithmically against the time (fig. 1) The

Table 1

Tissue distribution of dicoumarol in female rats fter ntravenous injection of 5 2 mg. Concentrations µg/g of wet tissue weight, except plasma concentrations, in µg/ml. (+) indicates amounts too small to be determined.

Organ tissue or fluid	Hours after injection of dicoumarol							
	1	3	6	12	18	24	36	48
Plasma	140 3	1[illegible]6.4	83 6	59 6	44 5	38 4	9.07	3 85
Liver	61 3	57 6	40 8	17	10 8	8 [illegible]9	4 82	3 05
Kidney	28.5	24 7	14 7	17 0	6 07	5 55	+	+
Lung	31.5	27 7	22.2	12.6	8 54	8 41	+	+
Heart	[illegible]3 1	19 6	14 0	5 47	6 90	3 99	+	+
Skin	17 7	20 3	13 5	6.92	4 67	4.26	+	+
Thyroid	–		16.0	7 08	3 31	7 82	+	+
Spleen	–	–	5 75	5 39	2 72	5 69	+	+
Muscle	–	–	10.9	3 63	[illegible].77	5 08	+	+
Brain	–	–	[illegible].02	1 99	1 33	+	+	+

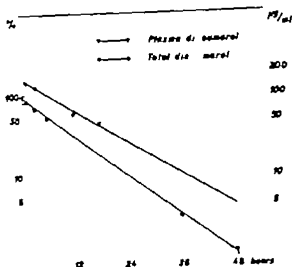


Fig. 1. Falls in plasma dicoumarol concentrations and of total amounts of dicoumarol recovered from female rats after intravenous administration (3.2 mg). Concentrations in μg/ml, total dicoumarol as percentage of dose injected.

plots closely fitted a straight line. The regression line drawn was obtained by the method of the least squares, and the half life on the decay-curve was calculated as 7.6 hours. The observed rate of disappearance of dicoumarol from rats is consistent with a first order reaction for the excretion or metabolism (or both) of the drug.

Rate of disappearance of dicoumarol from the plasma of rats. A logarithmic plot of the plasma concentrations of dicoumarol against the time from the intravenous injection is also shown in fig. 1. The regression line, also obtained by the method of the least squares, is nearly parallel with that shown for total dicoumarol, and the half-life of plasma dicoumarol was calculated as 9.2 hours.

Excretion of unchanged dicoumarol. Only small amounts of unchanged dicoumarol could be recovered from the urines of the individual rats, and quantitative determinations were therefore uncertain. Faeces too contained little unchanged drug, and the determinations were made further uncertain because of the short time for collection elapsing between injection and killing.

Metabolism of dicoumarol. After the administration of dicoumarol to the rats, paper chromatography of tissues, urine and faeces revealed the appearance of a substance with R_f-value of 0.55 (solvent: n-butanol – aqueous ammonia): on treatment with diazotized sulphanilic acid, a colour appeared like that obtained with dicoumarol. One spot, eluted from a chromatogram with 0.1 M HCl (5 ml), showed a spectrum nearly

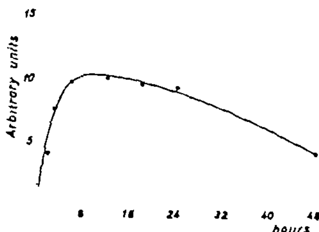


Fig. 2. Plasma levels, in arbitrary units, of unknown metabolite B-055 after intravenous administration of dicoumarol (5.2 mg) to female rats. The metabolite, identified chromatographically by R_F-value and colour determined by paper chromatography in essentially the same way as dicoumarol.

identical with that exhibited by the azo-dye of dicoumarol with a peak absorption at 415 mμ The absorbance of the unknown substance at 415 mμ (corrected for the blank value) was proportional to the amount of substance applied to a chromatogram. On this basis, the relative concentrations of the unknown substance in plasma in a series of rats was determined by essentially the same method as that used for dicoumarol. However the extraction from plasma was carried out at strongly acid pH The curve obtained (fig. 2) indicates that the plasma level of the metabolite, provisionally designated B-055 increased in the first 8 to 12 hours after intravenous administration of dicoumarol and then slowly decreased.

Discussion

The tissue distribution of dicoumarol observed by us in rats agrees well with the distribution of oral dicoumarol in rats, as described by HAUSNER *et al* (1951) and by GREEN *et al* (1956) despite the fact that different methods for determining dicoumarol were employed in these investigations The high level of liver dicoumarol compared with the low levels of brain, muscle, spleen, and erythrocytes, and the intermediate levels of kidney and lung, are thus consistent with the findings of these investigators and also with the distribution of dicoumarol found in dogs after intra

venous administration of the drug (WEINER *et al* 1950) It is therefore concluded that dicoumarol is unevenly distributed in animals, but tends to accumulate in the liver This also corresponds with the observations made by LEE *et al* (1950) on the fate of ^{14}C-labelled dicoumarol administered intravenously to mice and rabbits. They observed however that the level of radioactivity in the liver was constant for 16 hours in mice and for 3 days in rabbits, and they showed by the isotope dilution method that the radioactivity in the livers of rabbits at 24 hours represented unchanged dicoumarol. These observations are somewhat inconsistent with our finding that the level of liver dicoumarol tends to decline uniformly with that of plasma and that appreciable amounts of metabolites are present in the liver even 24 hours after intravenous administration of the drug. The differences between our observations and those of LEE *et al* may be ascribed to the difference in animal species studied, but the methods used for determining dicoumarol may also be of importance. An interesting observation made by LEE *et al.* was that injection of vitamin K favoured an increased displacement of dicoumarol from the liver tissues of mice and rabbits, and they suggested that the period of time during which dicoumarol is fixed in the liver is related to the anti-coagulant action of the drug. However GREEN *et al.*, in their studies on rats, were unable to demonstrate any influence of vitamin K on the tissue distribution of dicoumarol.

Only a small amount of information about the rate of disappearance of dicoumarol from whole animals is available. From the investigation by HAUSNER *et al* the half-life of the radioactivity in rats after oral administration of ^{14}C-labelled dicoumarol may be estimated at about 14 hours, in contrast with a half-life of 7 6 hours observed by us. However HAUSNER *et al* did not divide the radioactivity measured into that representing dicoumarol and that representing its metabolites, which could explain the observed difference in the half-life. In later experiments we have confirmed that the metabolite B-055 is radioactive if dicoumarol is labelled by ^{14}C in the same way as that used by HAUSNER *et al.* and administered to rats.

So far the chemical nature of the metabolites of dicoumarol, in both man and animals, remains unknown. HAUSNER *et al* have detected by paper chromatography 7 radioactive metabolites in urine and 4 in plasma after the oral administration of ^{14}C-labelled dicoumarol to rats, but no further information about these was given. In our study on rats we have also demonstrated the presence of several metabolites in urine and faeces, but one metabolite, which we have designated B-055 was predominated in both excreta and tissues, but the chemical nature of this main metabolite of dicoumarol in rats is at present unknown.

Summary

The distribution and elimination of dicoumarol administered intra venously to rats, have been studied by quantitative paper chromatography The levels of dicoumarol in the tissues decreased in accordance with the plasma level the highest amount of tissue dicoumarol being found in the liver the lowest in the brain erythrocytes and muscle. The rate of elimination followed the kinetics of a first order reaction and reflected the rate of metabolic transformation of the drug, only minute quantities being were excreted unchanged in the urine and faeces. Several metabolites were detected in the excreta by paper chromatography but one metabolite, designated B-055 was especially abundant here as in all the tissues examined

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The Determination of Absorption Rates from Rat Muscles an Experimental Approach to Kinetic Descriptions

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The true absorption rate of drugs can only be obtained by measuring their clearance rate from the local area of absorption, either continuously (for drugs labelled with γ-ray emitters) or by quantitative chemical or radiochemical analysis of the absorption zone at intervals. This principle has only occasionally been used for studying subcutaneous or muscular absorption, apart from the work devoted to comparative measurement of peripheral blood flow (KETY 1948 and others), though it has commonly been applied in studies on intestinal absorption.

To approach an analysis of the factors involved in the mechanism of drug absorption after intramuscular injection, the extensor quadriceps femoris muscle from rats was used for clearance studies. The carbohydrates mannitol, sucrose, inulin and dextran, for convenience labelled with ^{14}C or ^{3}H, were choosen as water-soluble and essentially lipid-insoluble test substances with great differences in molecular weights. They represent electroneutral, or essentially neutral, compounds, they are without pharmacodynamic effects (apart from a histamine liberating property of dextran), and they are believed not to be locally decomposed. The radioactivity may therefore be taken as a measure of how much of the parent compound remains at the injection site.

Our report covers a study of this method and its application to establishing whether or not water-soluble test compounds are absorbed through the capillary walls by diffusion. Further an analysis was attempted of the classic dogma, mentioned in most text books of pharmacology that there is an increase in absorption rate (and toxicity) of any given dose of a drug when injected parenterally in more concentrated solutions. The

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absorption rate was studied from constant volumes of solutions with various concentrations of test compounds, and in another series of experiments the injection volume was varied and the concentration of the injected test compound was kept constant.

The expression "absolute clearance" is used for the amount absorbed per unit of time "relative clearance" or simply "clearance" means the percentage of the total injected amount absorbed in unit time

Materials and Methods

Test substances

D-Mannitol-1-^{14}C specific activity (sp.a.) 6 mC/g, molecular weight (m.w.) 182.
Sucrose-^{14}C, sp.a. 36 or 67 mC/g, m.w 342.
Inulin-methoxy-^{3}H sp.a. 30 mC/g, m.w 3000–4000
Inulin-carboxyl-^{14}C, sp.a. 2.6 mC/g, m.w 3000–4000
Dextran-carboxyl-^{14}C, sp.a. 0 65 mC/g, m.w 60000–90000

The radiosucrose was obtained from The Radiochemical Centre, Amersham, England, the other radiochemicals from New England Nuclear Corp., Boston, USA. The injections of tritiated inulin contained 56 µC/ml, of the other substances 12–25µC/ml. If required, the concentration (mg/ml) was raised by adding ordinary unlabelled compound. When not otherwise stated, the injections were prepared in 0.9 / sodium chloride.

Animal experiments

Male albino rats, weighing 110–140 g, were tied in the supine position under sodium mebumal (pentobarbital) anaesthesia (8 mg/100 g rat intraperitoneally). Care was taken not to restrict their respiration or circulation by excessive stretching. They were kept warm by means of lamps. The body temperature was measured at the end of each experiment by inserting into the subclavian tissue a needle with a thermocouple connected to an electric thermometer (type TE 3 Elektrolaboratoriet Copenhagen) Animals showing an abnormal body temperature were discarded. A narrow incision was made through the skin over the thigh, from the middle of the groin down to 1 cm below *patella*. By blunt dissection the anterior femoral muscles were carefully exposed for their whole length and freed from overlying fatty tissue. Two ligatures were loosely tied around the *m extensor quadriceps femoris* just touching the underlying *femur* the one at the proximal, the other at the distal end of the muscle. They allowed the blood flow to the muscle to be cut off instantaneously at the end of the absorption period and facilitated isolation of the muscle. A short thin cannula with a short bevel (V2A 0.45 × 10) connected to an Agla micrometer syringe was inserted some few mm above the *ligamentum patellae* and the desired volume (4–64, usually 16 µl) was injected into the middle of the right extensor It was essential to redraw the cannula slowly to avoid leakage. The incision in the skin was rapidly closed, to avoid evaporation and cooling. At the end of the absorption period the proximal and then the distal ligatures were tightly tied. The *ligamentum patellae* was cut by tearing both ligatures together in the proximal direction the muscle could be pulled free of the *femur* without bleeding. The excised muscles usually weighed 1 0–1 6 g and had a constant specific gravity of 1 0.

Chemical procedure

The muscles were allowed to swell overnight in 0.5 N sodium hydroxide 3 times the volume of the muscle, then dissolved by heating to 90–95° with intermittent vigorous shaking in glass stoppered flasks for about 30 min. When cooled, the yellow turbid solution was treated with a 10 % solution of $ZnSO_4.7H_2O$ (cf. SOMOGYI 1930) with vigorous shaking. After centrifugation, 100–200 µl of the clear colorless supernatant were mixed with 4.00 ml of a liquid scintillation medium (BRAY 1960). A small amount of flocculent precipitate was allowed to settle, and the radioactivity was measured in a IDL Triton Scintillation Counter type 6012 (Isotope Developments Ltd. England). After subtracting the background radiation, the radioactivity of the total sample was calculated. Recirculation of radioactivity was determined in the left side extensors at various stages of the different absorption experiments and always found to be considerably below 1 % of the amount injected and no correction was applied for this. The efficiency of counting ^{14}C was about 60 % as estimated by means of an internal ^{14}C-standard.

The residual carbohydrate in a sample was expressed as the percentage of the amount injected. On each experimental day 4 standards, consisting of "blank" muscles (often the left extensor) were injected after isolation with the standard dose and put through the procedure described. The mean count of these four standards gave the amount injected. By keeping a fixed ratio between muscle weights and volumes of reagent, and by taking the standards through the procedure parallel with the samples, any effect on the result from the procedure should be eliminated.

Comments to Method

Effectiveness of ligation

To define the length of the absorption period exactly it is important that the local circulation to the muscle be abolished instantaneously. This was secured by ligation of the muscle (table 1), as no significant clearance was found from muscles injected with radiosucrose after ligation.

Table 1

Effect of Ligaturing upon Absorption.

The ligatured right m. extensor quadriceps femoris of 4 rats were injected with radiosucrose (0.35 mg/ml, about 12.5 µC/ml) and removed after 5 minutes. The left extensors were similarly injected after removal and served as controls.

Recovered radioactivity counts per minute 10^{-3}

	Ligatured muscles	Control muscles
	188	216
	239	226
	244	242
	260	248
Mean ± S.E.M.	233 ± 16	233 ± 7

Effect of the procedure on counting efficiency

If quenching was varying with the degree of hydrolysis, the counting efficiency might vary. To exclude this as a source of error, pooled radioactive muscles were allowed to swell in sodium hydroxide for 4 days at room temperature. They could then be "dissolved" after vigorous shaking. Measured portions of the "solution" heated at 100° for 0 to 45 minutes had identical counting rates (the results of this experiment are not presented).

Carboxyl groups and absorption rate

The ionisable carboxyl groups added to dextran and inulin might alter the adsorption and binding of the compounds to tissue constituents, and thereby influence the absorption rates of the compounds. However, this appears unlikely from experiments with the inulins. Inulin-carboxyl-^{14}C and inulin methoxy-^{3}H were prepared from the same unlabelled material (New England Nuclear Corp., personal communication). The ^{14}C compound has a carboxyl group at the end of the inulin chain. In the tritiated compound methyl groups have been added to a small proportion

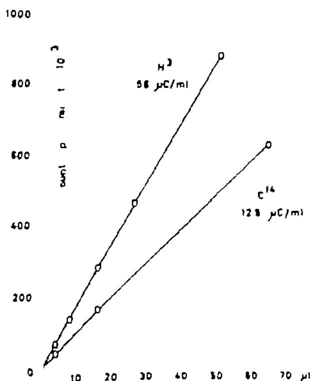


Fig. 1. Samples of m. extensor quadriceps femoris were removed and injected with either inulin-carboxyl ^{14}C (solution containing 12.5 µC/ml) or inulin-methoxy-^{3}H (56 µC/ml) and subjected to the chemical procedure described in text.
Abscissa: Injection volume (µl).
Ordinate: Counts per minute · 10^{-3}.
Each point is the mean for 3–4 muscle samples.

of the free carboxyl groups along the chain. Both procedures thus involve only small alterations in molecular size unless the ionised carboxyl group had a marked influence, the physiochemical properties of the parent compound would still largely determine absorption rates. This assumption seems verified by the similar absorption rates for the two inulins (table 2). Probably the same is true for dextran-carboxyl ^{14}C, which compared to the parent compound is relatively less labelled than inulin-carboxyl-^{14}C.

Reliability of standards.

As the counting efficiency tended to vary from one day to the other daily standards were prepared. In a preliminary experiment, 10 isolated muscles received 16 µl, 10 others 50 µl ^{14}C-inulin-injections. The standard deviations were 5 7 / and 3 9 /‰ respectively According to this, the mean for 4 standards should usually be a satisfactory reference amount. Moreover to minimise systematic error in the percentage residual amounts due to errors in the reference material, the absorption experiments on each substance were preferably spread over more than one day The standard curves were linear in the range studied (fig. 1)

Results

Molecular weight

The percentage amounts of mannitol sucrose, inulin and dextran remaining at various times after injection of 16 µl solutions containing

Table 2

Effect of Molecular size upon Absorption Rate.
16 µl of the solutions listed below with N Cl added to physiological isotonicity were injected. Results are given as mean percentage remaining amounts ± S E.M. Figures in brackets indicate the number of experiments.

	5 min	15 min.	30 min	60 min.
Mannitol 2.1–4 2 mg/ml	32.8 ± 2 4 (7)	9 7 ± 1 0 (7)	3 1 ± 0.1 (6)	
Sucrose 8 19 mg/ml	37.9 ± 4.5 (4)	14 9 ± 2.0 (4)	6.7 ± 0.4 (4)	
Inulin-carboxyl-^{14}C 4.8 mg/ml	84.6 ± 2.1 (4)	42.4 ± 3.5 (4)	20.5 ± 1 4 (4)	
Inulin-methoxy-^{3}H 1.9 mg/ml	77 9 ± 1 7 (4)	56.6 ± 3 8 (4)	28.5 ± 0.4 (4)	
Dextran 21 mg/ml	92.7 ± 2.4 (5)	81.3 ± 1.2 (13)	77.3 ± 2.7 (4)	74.4 ± 2.8 (5)

each of the carbohydrates in tracer concentrations, and sodium chloride added to physiological isotonicity are shown in table 2. Another presentation of these results is given in fig. 2 on a semilogarithmic plot, to show the relative slopes and the curvatures of the clearance curves. Whereas for all test compounds the amounts remaining at the injection site were determined after 5 15 and 30 minutes, only dextran was introduced in a 60 minute experiment because of its slow clearance. A decrease in absorption rate was found with increasing molecular size of the carbohydrates.

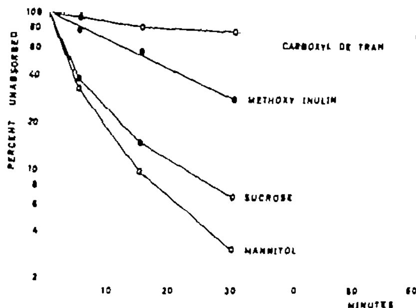


Fig 2. Effect of molecular weight on absorption rate.
Abscisse Time (minutes)
Ordinate Percentage of injected amount unabsorbed Logarithmic plot.
The curves are drawn on the basis of figures contained in table 2.

Varying concentrations

To allow comparison of the clearance rate with the molecular size of the test compound this rate should be unaffected by the concentrations of tracer compound in the injected solution Table 3 shows the percentage amounts of ^{14}C sucrose remaining 5 and 15 minutes after injections of 16 µl solutions containing from 0.19 up to 92 mg/ml The sucrose solutions containing 0 19 0 32–0 38 and 9 6 mg/ml were all prepared in saline (0 9 %) The clearance rates (relative clearance) of sucrose from these solutions were essentially identical as no statistically significant difference exists between the percentage amounts remaining of any of these solutions at similar times after injection The clearance rate from the solution containing 92 mg/ml sucrose seems somewhat lower as indicated by the significantly higher residual 5 minutes after the injection

This injection solution, however owed its physiological isotonicity only to the sucrose contents, sodium chloride having not been added.

Table 3

Effect of Concentration upon Absorption Rate.

16 µl of sucrose solutions, prepared either in 0.9 % sodium chloride (0.19 mg/ml–9.6 mg/ml) or in water alone (92 mg/ml), were injected. The results are given as mean percentage remaining amounts ± S.E.M. Figures in brackets indicate number of experiments.

	Concentration of sucrose, mg/ml			
	0.19	0.32–0.38	9.6	92
5 min.	37.9 ± 4.5 (4)	38.8 ± 2.3 (15)	42.7 ± 1.9 (4)	51.1 ± 2.7 (7)
15 min.	14.9 ± 2.0 (4)	14.1 ± 1.5 (9)	12.3 ± 1.7 (4)	

Varying injection volume

Though altering the concentration of the injected solutions did not particularly affect the relative clearance, the injected volume had an important effect for the rate of absorption. This has been demonstrated in experiments in which solutions containing constant concentrations of mannitol or sucrose were injected in various volumes into the muscle.

For sucrose the percentages of the injected dose remaining after 4, 16 and 64 µl of a solution containing 0.32–0.38 mg/ml were 21.5, 38.8 and 46.3 %, respectively 5 minutes after injection 15 minutes after the injection the comparable percentages were 4.3 14.1 and 18.6 % (cf table 4 and 3).

For mannitol (2.1 mg/ml) the percentages of the injected doses remaining were examined for injection volumes of 6, 16 and 42 µl. After 5 minutes 27.4 32.8 and 38.8 %, respectively were left at the injection site (table 4 and 2) 10 minutes after the injection of 6 and 42 µl, only 8.2 and 25.0 % were left, respectively (table 4).

Discussion

When an injection is performed, the tissue will be disrupted to a degree determined by the volume of injected fluid, resulting in local trauma. This trauma and the introduction of a fluid unphysiological in ion composition (even though isosmotic) may provoke liberation of vasoactive compounds in the tissue, such as histamine and 5-hydroxytrypta-

Table 4

Effect of Volume upon Absorption Rate.
6 and 42 µl of a mannitol solution (2.1 mg/ml) 4 and 64 µl of a sucrose solution (0.38 mg/ml) were injected. The results are given as mean percentage remaining amounts ± S.E.M
Figures in brackets indicate number of experiments.

		2½ min.	5 min.	10 min., Mannitol 15 min., Sucrose
Mannitol	6 µl	36 6 ± 3 6 (6)	27 4 ± 3 5 (6)	8.2 ± 1 6 (4)
	42 µl	–	38.8 ± 2.6 (3)	25 0 ± 2.3 (3)
Sucrose	4 µl	–	21 5 ± 8.2 (4)	4.3 ± 1.3 (4)
	64 µl	–	46.3 ± 3 9 (4)	18.6 ± 1 4 (4)

mine (cf SCHOU 1961) Further the injected drug may itself possess the property of liberating these compounds or may by some other (specific) pharmacodynamic action affect the capillaries and the peripheral blood flow Ionized compounds may become adsorbed or bound within the injection zone to immobile tissue components carrying the opposite charge This binding may take place within the pores of the capillary wall thereby reducing the effective watery diffusion area. Whereas lipid-soluble substances are able to pass the entire capillary wall lipid insoluble substances can penetrate only through the pores Hence the lipid/water partition coefficient and the ionization constant of a compound are of importance to the absorption rate

In sum, many factors widely differing in nature may influence the absorption of an injected compound In our experiments we have attempted to keep constant as many of these as possible

As pointed out in the introduction the substances had a high water/lipid partition ratio and they were neutral, or essentially neutral, and pharmacologically inert apart from the histamine-liberating property of dextran Thus, interaction from ionization lipid-solubility and pharmacological activity (except for dextran) can be left out of account Further the use of labelled compounds permitted low and osmotically insignificant concentrations to be applied Isotonicity could therefore be obtained in all experiments by adding sodium chloride at the same concentration (except in the experiment with isotonic sucrose solution) Hence the composition of the various solutions can be considered to be unphysiological to the same extent.

Molecular weight

The experiments show the molecular weight to be an important variable, the clearance rates being inversely related to it. The absorption from muscles may proceed directly into the blood or indirectly through the lymph. Direct transport, through the walls of the blood capillaries, is usually considered by far the more important (for a discussion, cf SCHOU 1961).

The blood capillary wall behaves as a lipoid-pore layer allowing lipid soluble substances to penetrate readily through the entire surface. In contrast, water-soluble compounds appear to pass through water filled pores, whose total cross-section comprises less than 0.2 / of the area of the capillary surface (cf review by SCHANKER 1962) This passage is commonly supposed to proceed essentially by diffusion. We consider that our experiments have supported this assumption. This comes from comparing the clearance rates of the four carbohydrates with their free diffusion coefficients. During the first 5 minutes after injection, the cleared fractions of mannitol, sucrose, the inulins and dextran were about 0 7 0.6, 0.2 and 0 07 respectively This corresponds fairly well with the diffusion coefficients, which are (D 10⁵ 37°) for mannitol 0.87 (INTERNATIONAL CRITICAL TABLES 1929 by interpolation), for sucrose 0.75 and inulin 0.21 (RAPPENHEIMER, RENKIN & BORRERO 1951) and for the 60000 m.w fraction of dextran probably about 0 05 (cf STAVRI 1961) Unless diffusion is the main process, the correlation would be difficult to explain. Thus, if the absorption was caused predominantly by filtration, the various sized substances would be expected to clear the tissue at similar rates unrelated to their diffusion coefficients.

The cleared quantities (the amounts disappearing during the period expressed as fractions of those existing at its beginning) during the 5–15 minutes period showed a similar though not this close, correlation. Inulin (there is evidence that the carboxyl-compound was slightly deteriorated, which might have affected its clearance at the later stage) and in particular dextran appeared to pass out more readily than was to be expected.

The correlation pointed out above deserves a more thorough examination. Further discussion of the matter is being published (SUND & SCHOU 1965).

The somewhat large initial clearance rate of dextran appears a little surprising in view of the relative dimensions of the dextran molecules and the pores of the blood capillary wall. The R_{ES} value of dextran of m.w 60000 seems to be about 40 Å (cf STAVRI 1961), and the mean pore radius according to PAPPENHEIMER, RENKIN & BORRERO (1951) is 30 Å. This suggests that the absorption of dextran would be greatly retarded, as only

Table 4

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6 and 42 μl of a mannitol solution (2.1 mg/ml) 4 and 64 μl of a sucrose solution (0.38 mg/ml) were injected. The results are given as mean percentage remaining amounts ±S.E.M. Figures in brackets indicate number of experiments.

		2½ min.	5 min.	10 min., Mannitol 15 min., Sucrose
Mannitol	6 μl	36.6 ± 3.6 (6)	27.4 ± 3.5 (6)	8.2 ± 1.6 (4)
	42 μl		38.8 ± 2.6 (3)	25.0 ± 2.3 (3)
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a fraction of the pores would be large enough for the molecules to pass through The lymph capillary wall (if such a wall exists) leaks more than the blood capillary wall Therefore drainage through the lymph could be of relatively greater importance for the absorption of large than of smaller molecules On the other hand the ratio of the amount cleared within a certain period to the diffusion coefficient had for dextran about the same value as for the other compounds at least it was no smaller This indicates that absorption proceeds along the same route for all substances Possible explanations for the phenomenon then appear to be 1 The preparation of dextran contains a fairly large fraction of a smaller m w than that stated (60000–90000) 2 The capillary permeability of rats is greater than indicated above. It might further have become increased owing to the anaesthesia the operative procedure, the injection or the action of histamine liberated by dextran. Rat muscles contain histamine (FELDBERG 1956)

Curvature of the clearance curves

If the absorption process could be described by a single exponential equation, a semilogarithmic plot of the residual amounts should yield straight lines as pointed out by KETY (1949) There is, however a tendency to curvature in our experiments (fig. 2) as also noted by KETY (1954) for the clearance of ^{24}NaCl injected into muscles To explain the curvature he suggested the existence of rapidly and slowly circulated areas within the same tissue, which did not contribute constant in proportion to the net absorption as time passed This point possibly deserves further consideration in connection with our experiments as *m extensor quadriceps femoris* is a group of intermingled muscles.

Other factors also have an effect on the "clearance constant" (KETY 1949), and their quantitative influences tend to change with time The effects of some tends to enhance absorption of others the opposite. Complete compensation cannot be expected Some of these effects are spreading within the tissue of the injected substance incomplete mixing ("stirring) within the "injection pool change in the net resistance to diffusion variation in concentration due to absorption of water varying recirculation of the absorbed substance by the arterial blood.

It has been suggested that mere injection into the tissues provokes an unspecific self-depression due to local tissue trauma causing a liberation of histamine and 5-hydroxytryptamine (cf SCHOU 1961) If this is so and the vasoactive substances are progressively released such a mechanism could add to the curvature. The existence of this mechanism is, however

open to dispute, for locally injected histamine has also been claimed to accelerate clearance rates (McGIRR 1957).

The absorption graph for dextran appears more curved than those for the other substances. This is probably mainly due to the heterogeneity of the material, but a "specific" self-depression might cooperate, as dextran is a histamine-liberator

Varying concentrations

Sucrose had practically a constant clearance rate for solutions containing 0.19 mg/ml (0.55 mM/l) up to 9 6 mg/ml (28 mM/l), as evident from the residuals 5 and 15 minutes after injection (table 3). This behaviour is taken as representative for the class of pharmacologically inert, neutral water-soluble substances. It is concluded that the concentrations do not particularly influence absorption rates of these compounds, in so far as only osmotically insignificant amounts are applied. In contrast, the absorption rate of e.g. atropine, depends markedly upon concentration (SUND & SCHOU 1964)

When a solution was injected with added sucrose as the only component to obtain isotonicity the absorption rate appears to be decreased (table 3). This may be explained by a greater self-depression of the absorption from this solution. As stated above, the other solutions injected are considered to be unphysiological to the same extent, so that any final self-depression should be identical. A replacement of sodium chloride by sucrose may further depress smooth muscle (HOLMAN 1957), affecting the vascular muscles and thereby the capillary flow Also diffusion coefficients decrease to some extent with increasing concentrations.

Varying injection volume

The absorption rate is affected to a significant degree when the injection volume is altered (table 4) This has previously been demonstrated for the strong electrolyte sodium chloride (WARNER *et al* 1953) and for the alkaloid atropine (SCHRIFTMAN & KONDRITZER 1957). Our experiments with the neutral, pharmacologically inert sugars mannitol and sucrose thus show that the phenomenon is general. That the relative clearance is inversely related to the injection volume was attributed by WARNER *et al* (1953) to a relatively limited capillary bed active to absorption, to a disproportionate ratio between increase in surface area and increase in volume and to the relatively long diffusion path to be travelled by molecules from the centre of the injection site.

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Acknowledgements.

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Conclusion

Absorption is the first critical step in the sequence after administration of a drug, and a study of its kinetics deserves close attention. Until now absorption from subcutaneous or intramuscular sites has mainly been studied rather indirectly by various procedures (cf SCHOU 1961) These at best allow qualitative and comparative statements to be made about absorption rates, but much information may become lost, and the true rates cannot be obtained This appears possibly only by determining the local amount of drug remaining.

The results reported here serve to show that this principle, when applied to studies of intramuscular and possibly also subcutaneous absorption, yields a quantitative method on small animals. The direct information supplied by this principle can be expected to become of value in various respects Some examples are 1 To assess more exact the relative significance of the forces that operate absorption 2 to establish quantitatively the effect of ionization, relative lipid water solubility and pharmacological activity upon absorption rates (for this purpose, comparison with a neutral lipid-insoluble and pharmacologically inert compound, e.g. a carbohydrate of suitable molecular size, would be helpful 3 to investigate further the effect of various pharmacological treatments on absorption rates when the evidence conflicts e g. the effects of locally injected histamine and of oestrogen treatment (cf SCHOU 1961)

Summary

Mannitol, sucrose, inulin and dextran, for convenience labelled with ^{14}C and ^{3}H were dissolved in 0 9 % sodium chloride and injected into the *m extensor quadriceps femoris* of anaesthetized rats After various times the muscles were removed, digested and analysed for the percentage residual radioactivity by liquid scintillation technique The absorption rates thus obtained were inversely related to molecular weight, showing diffusion to be a main driving force for the absorption. The effect of varying injection volume and concentration was obtained in experiments with mannitol and sucrose. The absorption rates increased when the volume was decreased, but were identical over a range of concentration (0 55 to 28 mM). From isotonic solutions containing sucrose only the rate appeared somewhat reduced Possible reasons for curvature in a semilogarithmic plot of residual amounts against time are discussed. The advantage of studying the kinetics of absorption by determining the quantities unabsorbed is stressed.

Silicagel- G plates (½ mm thick), which had been activated by heating to 110°C for one hour and stored on silicagel, were used for thin-layer chromatography

A specimen of human liver that had been minced and stored for three years at 4–5° was examined. No poison of any kind had been demonstrated in the liver by the original medico-legal chemical analysis performed in this Institute. The liver presented no macroscopical indications of putrefaction. An extract was made from the liver by the method described below

In an M.S.E. homogeniser 25 g of liver tissue were homogenized with approximately 5 g of crystalline tartaric acid. The homogenate was extracted three times, each time with a three times its volume of ether The combined ether extracts were dried with anhydrous sodium sulphate, filtered and evaporated on a steam bath to 20 ml. The residue was extracted twice, each time with 15 ml of 0.5 N-NaOH. To the combined aqueous phases were added 2 ml of 3 M KH_2PO_4, as well as 2 N-H_2SO_4, pH 6.8. Extractions were now performed three times, each time with equal volumes of ether The combined dried and filtered ether extracts were distilled to a volume of 1–2 ml. The residue was boiled for approximately one minute with 30 ml of 0.1 N-H_2SO_4. The sulphuric acid solution was cooled under the tap to approx. 20 and filtered. The filtrate was extracted with 5 ml of chloroform which was discarded. The aqueous phase was extracted three times, each time with equal volume of ether The total dried and filtered ether extracts were evaporated at approx. 50° to dryness. According to the procedure described by EDER (1927), the residue was sublimed at about 80° under a pressure of approx. 5 mm Hg. The sublimate, weighing approx. 1 mg, consisted of fine white crystalline needles with an aromatic smell

Results

The melting point of the substance isolated by the above mentioned extraction procedure was 90 5° (corrected) its spectra in the infrared and the ultraviolet regions are recorded in figs. 1 and 2.

The constants obtained were found to be in good agreement with those mentioned in the literature for p-hydroxyphenylethanol. As it proved impossible to procure specimens of p-hydroxyphenylethanol, we have prepared the compound by two different methods, *viz* 1) a biochemical method in which yeast was caused to act on p-hydroxyphenylpyruvic acid (NEUBAUER & FROMHERZ 1910) and 2) a chemical method, by which KNO_2 was caused to act on tyramine (EHRLICH & PISTSCHIMUKA 1912).

A. *Preparation of p-hydroxyphenylethanol*

1. Biochemical method

To 200 mg of p-hydroxyphenylpyruvic acid puriss. (Fluka) were added 2 g of baker's yeast and 100 ml of 0.1 M-KH_2PO_4, pH 7.0. The mixture was stored for 48 hours at approx. 20° The p-hydroxyphenylethanol formed was extracted by the procedure described above for its isolation from liver tissue. The residue was subjected to thin-layer chromatographic purification. A mixture of ethyl acetate, isopropanol and water in the proportion 65.24 11 was used as solvent (STAHL & KALTENBACH

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From the Section of Forensic Chemistry (Fanny Halstrom, Ph.D) Department of Pharmacology University of Copenhagen (Professor Knud O Moller M D.)

Interfering Substances by Determination of Poisons in Autopsy Material.
I p-Hydroxyphenylethanol

By

Bent Kempe

(Received July 9 1964)

In chemical determinations performed on autopsy material – especially on putrefied specimens – impurities will often be extracted in amounts large relative to the total amount of poisonous substance isolated

In determinations such as spectrophotometry chromatography polarography *etc* some of these impurities may behave like the assumed poisonous substance under investigation or like its metabolites this state of affairs can lead to an erroneous analyses, perhaps with serious consequences It is therefore desirable that substances compromising the deter mination of the poisonous substance under consideration should be known.

The study recorded here presents a detailed examination of a frequently occurring impurity p-hydroxyphenylethanol Isolation and identification of the substance will be described below and methods reported by which the substance can be separated from 5 5-substituted barbituric acids, ex tracted in the same phase.

Technique

General technique.

The melting points were determined by means of the micro-melting point apparatus (HALSTROM 1940).

The pH was determined in a pH meter (type PHM 24c Radiometer) fitted with glass electrode C.

The spectra in the ultraviolet region were determined for aqueous solutions by means of a self-recording Beckman spectrophotometer DK 2.

The spectra in the infrared range were determined for KBr tablets by a Perkin Elmer IR-spectrophotometer model 21

Silicagel- G plates (½ mm thick), which had been activated by heating to 110°C for one hour and stored on silicagel, were used for thin-layer chromatography

A specimen of human liver that had been minced and stored for three years at 4–5° was examined. No poison of any kind had been demonstrated in the liver by the original medico-legal chemical analysis performed in this Institute. The liver presented no macroscopical indications of putrefaction. An extract was made from the liver by the method described below

In an M.S.E. homogenizer 25 g of liver tissue were homogenized with approximately 5 g of crystalline tartaric acid. The homogenate was extracted three times, each time with a three times its volume of ether The combined ether extracts were dried with anhydrous sodium sulphate, filtered and evaporated on a steam bath to 20 ml. The residue was extracted twice, each time with 15 ml of 0.5 N-NaOH. To the combined aqueous phases were added 2 ml of 3 M-KH_2PO_4, as well as 2 N-H_2SO_4, pH 6.8 Extractions were now performed three times, each time with equal volumes of ether. The combined dried and filtered ether extracts were distilled to a volume of 1-2 ml. The residue was boiled for approximately one minute with 30 ml of 0.1 N-H_2SO_4. The sulphuric acid solution was cooled under the tap to approx. 20° and filtered. The filtrate was extracted with 5 ml of chloroform which was discarded. The aqueous phase was extracted three times, each time with equal volume of ether The total dried and filtered ether extracts were evaporated at approx. 50 to dryness. According to the procedure described by EDER (1922), the residue was sublimed at about 80° under a pressure of approx. 5 mm Hg. The sublimate, weighing approx. 1 mg, consisted of fine white crystalline needles with an aromatic smell

Results

The melting point of the substance isolated by the above mentioned extraction procedure was 90.5° (corrected) Its spectra in the infrared and the ultraviolet regions are recorded in figs. 1 and 2.

The constants obtained were found to be in good agreement with those mentioned in the literature for p-hydroxyphenylethanol. As it proved impossible to procure specimens of p-hydroxyphenylethanol, we have prepared the compound by two different methods, viz 1) a biochemical method in which yeast was caused to act on p-hydroxyphenylpyruvic acid (NEUBAUER & FROMHERZ 1910) and 2) a chemical method, by which KNO_2 was caused to act on tyramine (EHRLICH & PISTSCHIMUKA 1912).

A. *Preparation of p-hydroxyphenylethanol*

1. Biochemical method

To 200 mg of p-hydroxyphenylpyruvic acid purum. (Fluka) were added 2 g of baker's yeast and 100 ml of 0.1 M KH_2PO_4, pH 7.0. The mixture was stored for 48 hours at approx. 20° The p-hydroxyphenylethanol formed was extracted by the procedure described above for its isolation from liver tissue. The residue was subjected to thin-layer chromatographic purification. A mixture of ethyl acetate, isopropanol and water, in the proportion 65 24 11 was used as solvent (STAHL & KALTENBACH

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From the Section of Forensic Chemistry (Fanny Halstrom, Ph.D), Department of Pharmacology University of Copenhagen (Professor Knud O Moller M D.)

Interfering Substances by Determination of Poisons in Autopsy Material
I p-Hydroxyphenylethanol

By

Bent Kempe

(Received July 9 1964)

In chemical determinations performed on autopsy material – especially on putrefied specimens – impurities will often be extracted in amounts large relative to the total amount of poisonous substance isolated

In determinations such as spectrophotometry chromatography polarography *etc* some of these impurities may behave like the assumed poisonous substance under investigation or like its metabolites this state of affairs can lead to an erroneous analyses perhaps with serious consequences It is therefore desirable that substances compromising the determination of the poisonous substance under consideration should be known

The study recorded here presents a detailed examination of a frequently occurring impurity p-hydroxyphenylethanol Isolation and identification of the substance will be described below and methods reported by which the substance can be separated from 5 5-substituted barbituric acids, extracted in the same phase

Technique

General technique

The melting points were determined by means of the micro-melting point apparatus (HALSTROM 1940).

The pH was determined in a pH-meter (type PHM 24c Radiometer) fitted with glass electrode C.

The spectra in the ultraviolet region were determined for aqueous solutions by means of a self-recording Beckman spectrophotometer DK –

The spectra in the infrared range were determined for KBr tablets by a Perkin Elmer IR-spectrophotometer model 21

1961). The plate was saturated for 20 minutes, then developed for 60 minutes. Approximately one tenth of the developed and dried plate was sprayed with Gerngross's reagent for p-substituted phenols (GERNGROSS, VOSS & HERFELD 1933). On subsequent heating to 60° a red strip appeared with an R_F-value of approx. 0.85. A corresponding area on the remaining nine-tenths of the plate was scraped off and extracted by means of 10 ml of methanol with magnetic stirring for 15 minutes. The extract was evaporated to dryness, and the residue sublimed as described above. The sublimate, estimated to weigh 20 mg, consisted of fine white crystalline needles with an aromatic smell. Its melting point was 90.5° (corrected). The spectra in the infrared and ultraviolet regions were recorded. An elementary analysis gave the values 69.45 % C (69.53), 7.23 % H (7.29). The figures in brackets are the calculated percentages, corresponding to the composition $C_8H_{10}O_2$. On mixing the sublimate with the substance isolated from the liver we obtained a mixture melting point of 91° (corrected).

2. *Chemical method*:

A solution of 0.152 g KNO_2 in 10 ml of demineralized water is added to 0.2283 g tyramine, HCl (B.D.H.), which has an equivalent figure of 173,9 by calculation from titration of its chloride content. The pH of the mixture is adjusted to 7.0 with 0.1 N-HCl, and the mixture is boiled for two hours. After cooling under the tap the mixture is extracted three times, each time with three times its volume of ether. On evaporation of the dried ether extract, a yellow syrupy residue is formed (approx. 15 mg). The residue is sublimed at 90° at a pressure of 5 mm Hg. Approximately 2 mg of white crystalline sublimate of a pale yellowish colour and an aromatic smell is collected. The melting point of a sample was 90° (corr.). The spectra in the infrared and ultraviolet regions are recorded.

On comparing the figures for the crystalline substance, isolated from putrefied liver with the specially prepared p-hydroxyphenylethanol, we found the two substances to be identical. In an aqueous solution at either acid pH (2) or basic pH (10), the p-hydroxyphenylethanol was found to have the same absorption at a wavelength of approx. 240 mμ as have barbiturates. In the contrast to that of the 5.5-substituted barbituric acids, the maximum of p-hydroxyphenylethanol at the wavelength 240 mμ does not shift when the pH is changed from 10.8 to 13. This, however, cannot be used for quantitative determination in mixtures, and an attempt has therefore been made to separate the substances.

B. *Separation of the substance p-oxyphenylethanol from barbituric acids.*

A solution of the p-hydroxyphenylethanol in 0.1 N-H_2SO_4 is shaken by hand for two minutes with an equal volume of chloroform or ether in a 25 ml glass tube fitted with a glass stopper; the distribution coefficient of the substance at room temperature is then determined by calculating the result from the spectrophotometric readings of the aqueous phase in the wavelength range 250–350 mμ. The distribution coefficients found were 0.1 N-H_2SO_4/ether = 0.78, and 0.1 N H_2SO_4/chloroform = 9.1.

The p-hydroxyphenylethanol gives no precipitate with the acid mercuric sulphate reagent used by FLEURY (1925) for precipitating 5.5-substituted

E
1.0
0.5
0.4
0.3
0.2
0.1

E
1.0
0.5
0.4
0.3
0.2
0.1

650 700 800 900 1000 1100 1200 1300 1400 1500 1600 1700 cm⁻¹

Fig. 1 Infrared spectra of the isolated substance (the upper curve) and p-hydroxyphenylethanol (the lower curve) in KBr

1.0

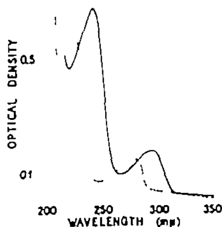


Fig. 2. Ultraviolet spectra of the isolated substance in 0.02 N borat buffer pH 10.6 ———— and in 0.1 N HCl - - - - - - - -

and pH 2.0 multiplied by the factor 4.1 equals the extinction at 240 mμ.

The barbiturates show no change of absorption at 292 mμ with changing pH, a fact which makes it possible to correct for the contribution of p-hydroxyphenylethanol in the spectrophotometric determination of barbiturates. The maximal content of barbiturates in an extract is determined by the difference of extinction at 292 mμ and at pH 10.8 and 2.0 multiplied by 4.1 substracted from the measured extinction at 240 mμ at pH 10.8 and 2.

Discussion

CURRY (1955) describes a "phenol-like" impurity that he observed during toxicologic analyses for barbiturates. His description of this impurity agrees with that found in the literature for p-hydroxyphenylethanol, though CURRY does not state the identity of the substance. It seems reasonable to assume, therefore, that what he described is identical with the p-hydroxyphenylethanol demonstrated in the work reported here.

CURRY reported that in paper chromatographic examinations, the impurity showed a blue fluorescence in ultraviolet light: this does not occur with purified p-hydroxyphenylethanol. The fluorescence must presumably have been caused by other impurities, for often on analysing organ material we have observed blue fluorescent regions with an R_F-value similar to that of the p-hydroxyphenylethanol.

Another impurity, *viz.* p-hydroxyphenylpropionic acid, has previously been isolated from bodies (NICKOLLS 1951, LYNCH 1951). The substance shows *an absorption curve in the ultraviolet similar to that of p-hydroxy*phenylethanol, but on being developed by the method of ALGERI & WALKER (see table 1), it has an R_F-value of 0.2 on Whatman paper No. 1. The substance is therefore easily separable from commonly occurring barbiturates.

Summary

One of the impurities frequently observed in chemical spectrophotometric determinations of 5.5-substituted barbiturates is identified as a phenol, *viz.* p-hydroxyphenylethanol. Methods are described by which this substance can be separated from barbiturates as well as methods by which spectrophotometric measurement of barbiturates can be corrected for contamination with p-hydroxyphenylethanol.

Table 1

R_F-value of p-hydroxyphenylethanol and some barbiturates on a) Whatman paper No. 1 and b) *activated silica gel-G plate (1 mm thick)* both developed by the ascending route with the upper phase of a mixture consisting of n butanol, 25 / NH_3, and water in the ratio 50 25 40 ml (ALGERI & WALKER, 1952) The lower phase of the mixture is used for saturating the paper and the plate for 90 minutes and 30 minutes, respectively

a)	p-Oxyphenylethanol	0.92
	p-Oxyphenylpropionic acid	0.20
	Aprobarbital	0 64
	Barbital	0.48
	Phenobarbital	0.57
	Pentobarbital	0 78
	Amobarbital	0.77
b)	p-Oxyphenylethanol	0 83
	p-Oxyphenylpropionic acid	0.28
	Aprobarbital	0.53
	Barbital	0.42
	Phenobarbital	0.42
	Pentobarbital	0 63
	Amobarbital	0.63

barbituric acids This method modified by REINERT (1928) has been used in Denmark since 1928 for purifying barbituric acid derivatives.

After chromatography by the method of ALGERI & WALKER (1952), the R_F-value of the p-hydroxyphenylethanol was found to be 0.9 (see table 1) on either Whatman paper No 1 or silicagel-G plates. For comparison the R_F-values of some barbituric acids are given in table 1 If examined in light of shortwave length (254 mμ) after spraying with 0 1 N NaOH p-hydroxyphenylethanol will appear on the paper chromatogram as a dark spot in a similar way to 5 5-substituted barbituric acids. In contrast to 5 5-substituted barbituric acids, however p-hydroxyphenyl ethanol cannot be developed by spraying with a mercuric sulphate reagent.

Our results support the conclusion that it is not possible to separate 5 5-substituted barbituric acids from the p-hydroxyphenylethanol by extraction a separation can however be performed either by precipitating the barbituric acids with mercuric sulphate reagent or by performing a paper or thin-layer chromatographic purification A simple calculation, based on spectrophotometric measurements, makes it even possible to determine directly the largest possible amount of barbituric acids that can be contained in a solution contaminated with p-hydroxyphenylethanol As mentioned above, p-hydroxyphenylethanol in alkaline solution has an absorption maximum at 292 mμ which shift to 275 mμ when the reaction is changed to pH2. Measurements on pure p-hydroxyphenylethanol have shown that the difference between the extinctions at 292 mμ at pH 10.8

and pH 2.0 multiplied by the factor 4.1 equals the extinction at 240 mμ.

The barbiturates show no change of absorption at 292 mμ with changing pH, a fact which makes it possible to correct for the contribution of p-hydroxyphenylethanol in the spectrophotometric determination of barbiturates. The maximal content of barbiturates in an extract is determined by the difference of extinction at 292 mμ and at pH 10.8 and 2.0 multiplied by 4.1 substracted from the measured extinction at 240 mμ at pH 10.8 and 2.

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Interfering Substances by Determination of Poisons in Autopsy Material. II. Tyramine

By

Bent Kaempe

(Received July 13, 1964)

In continuation of previous studies on breakdown products in putrefied autopsy material, this paper describes an analytical study of another frequently occurring impurity tyramine. Below its isolation and identification will be described, as well as methods by which it can by separated from morphine, when this is present in the same extraction phase.

Technique

The substance s melting point, pH and distribution coefficients are determined, its spectra in the infrared and ultraviolet regions are recorded, and plates for chromatography prepared by the procedures previously mentioned (Kaempe 1964).

A specimen of human liver which had been minced and stored for three years at 4-5° was the subject of the analyses. The original chemical analysis, performed in our Institute, did not demonstrate the presence of any poisonous substance in the body Death had presumably been caused by an acute kidney insufficiens. The liver showed pronounced macroscopic indications of fungal or bacterial action, or both, and an amino-like and putrefied smell. An extract was prepared from the liver by the procedure described below

For one hour 100 g of liver tissue, 400 ml of 96 % ethanol and 20 ml conc. HCl were heated on boiling water-bath. The hot mixture was centrifuged, and the clear supernatant decanted and neutralized with 8 N-NaOH The neutralized solution was evaporated on a water-bath, evaporation to dryness being avoided by suitable additions of water The cooled and filtered aqueous solution was extracted twice, each time with equal volumes of ether which were discarded. At approx. pH 2,2 N-H_2SO_4 was added two further extractions of the mixture were performed, each time with equal volumes of ether the ether phase was again discarded. Finally 10 ml of 0.5 M borate solution was added, and the pH was adjusted to 10.0 with 2 N-NaOH. After being saturated with crystalline sodium chloride, the mixture was extracted three times, each time with equal volumes of chloroform-isopropanol (3 + 1 /v). The total extracts

were dried with anhydrous sodium sulphate, filtered and evaporated to dryness on a water bath. The residue was dissolved in 20 ml of boiling 0 1 N HCl, cooled under the tap to approx. 20° and filtered. The filtrate was extracted as described above. The total dried and filtered chloroform-isopropanol extracts were evaporated to dryness on a water-bath. The yellowish crystalline residue was purified by thin-layer chromatography The solvent was the upper phase of a mixture of butanol, glacial acetic and water in the ratio 40 10 50 ml (PARTRIDGE & WESTALL 1948) The lower phase of the mixture was used for saturating the tank and the plate for 30 min. Chromatography was conducted for 90 min. One tenth of the developed and dried plate was sprayed with Gerngross's reagent. A red strip with an R_F-value of 0.5 was observed. The remaining region opposite the R_F-value 0.5 was scrapped off and eluted by 10 ml of methanol with magnetic stirring for 15 minutes. The eluate was evaporated to dryness on a water-bath. Two gram of a mixture of equal parts of sodium bicarbonate and sodium carbonate, and 1 ml water were added to the residue and extractions were performed 10 times, each time with 10 ml of chloroform. The total dried and filtered chloroform extracts were divided into two equal portions. Gaseous HCl was led to one half and both solutions were then evaporated on a water bath to dryness. The residue from the hydrogen chloride treated portion weighed approx. 0.05 g and consists of fine white crystals. The second residue weighing approximately the same, was amorphous and faintly yellow

Results

The corrected melting points of the base so isolated and of the HCl-salt were found to be 158 and 273 respectively The salt melted with decomposition

The spectra of the salt in the infrared and ultraviolet regions are recorded in figs. 1 and 2.

The values found were in agreement with those given in the literature for tyramine and tyramine – HCl, respectively A sample of tyramine-HCl puriss (Fluka) was purchased for comparative purposes. The sample showed a (corrected) melting point of 271 with decomposition a calculation based on the analysis of the chloride content by titration gave an equivalent weight of 175 9 The spectra in the ultraviolet and infrared regions were recorded. The corrected mixed melting point of the HCl-salt from the isolated substance and purchased tyramine, HCl were found to be 271 with decomposition

On comparing the values obtained for the HCl salt of the substance extracted from putrefied liver tissue with the tyramine-HCl, we found that the two substances were identical. As tyramine has two free positions ortho to the hydroxyl group it will be included in the polarographic morphine determination (BAGGESGAARD RASMUSSEN, HAHN & ILVER 1945) For experimental pharmacological analyses, some investigators at this institute have further developed the polarographic morphine determinations before measuring it, two of these investigators (PÆRREGAARD

Fig. 1. Infrared spectra of the isolated substance (the upper curve) and tyramine (the lower curve) in KBr

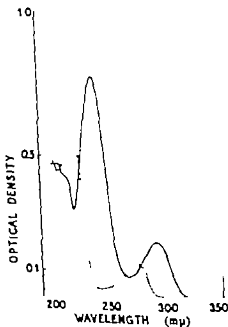


Fig. 2. Ultraviolet spectra of the isolated substance in 0.1 N NaOH ——— and in 0.1 N HCl ———

(1957) and MILTHERS (1959)) performed paper chromatographic purifications by the methods of JATZKEWITZ (1953) and BROSSI *et al* (system *A*) (1952), respectively. As polarography is used in chemical analyses for morphine, tyramine should be removed before the measurement. The two paper chromatographic systems mentioned do not permit a sufficiently clear separation and an attempt has been made to find another method for separating the two substances.

A *Separation of tyramine from morphine*

The distribution coefficient of tyramine is 8.3 in saturated aqueous bicarbonate solution at pH 8.2/chloroform-isopropanol (3 + 1 v/v) and 0.40 in a NaCl-saturated aqueous borate solution at pH 9.0/chloroform-isopropanol (3 + 1 v/v). As expected extractions with ether from equal volumes of tyramine-containing 0.5 N HCl or extractions with chloroform from equal volumes of tyramine-containing 0.5 N NaOH will hardly make the tyramine pass into the organic phase. By shaking one volume of iso-amyl alcohol with 5 volumes of boiling 2 N H_2SO_4 containing tyramine, the tyramine will likewise remain on the aqueous phase. The R_F-value of the tyramine is 0.06 on whatman No. 1 paper prepared with a buffer of approximately 0.1 M sodium phosphate, pH 6.3 after chromatography in BROSSI *et al.* system *A* (1955). Preparation of the paper with a phosphate buffer of pH 7.0 will not involve any appreciable change in R_F-value. Thin layer chromatography with PARTRIDGE & WESTALL's mixture (1948) gives an R_F-value of 0.5. The colour with FROHDE's (1866) reagent is a strong blue. (The R_F-values of morphine, showing a violet colour with Frohde's reagent, are seen in table 1.)

When isolating morphine in our Institute, we extract from a solution saturated with $NaHCO_3$ with large volumes of chloroform-isopropanol (3 + 1 v/v) (BAGGESGAARD RASMUSSEN & SCHOU 1928). By this procedure the distribution coefficient for tyramine is 8.3 and the extract will therefore be more or less contaminated with any tyramine that might be present. It is not possible to obtain a separation of morphine from tyramine

Table 1

R_F-value of tyramine and morphine on Whatman No. 1 paper prepared with an approx. 0.1 M phosphate buffer pH 6.3 by ascending chromatography with a mixture of amylene hydrate, di-n-butyl ether and water in the proportion 80 : 7 : 13 (BROSSI *et al.* 1955). With a phosphate buffer at pH 7.0 the R_F-values shown in column b are obtained.

a)	Tyramine	0.06
	Morphine	0.11
b)	Tyramine	0.1
	Morphine	0.5

Table 2

R_F-value of morphine and tyramine on ½ mm thick silica-G plate, activated by heating to 110° for one hour and developed with the upper phase of mixture of butanol, glacial acetic acid, water in the ratio 40:10:50 ml (PARTRIDGE & WESTALL 1948).

Morphine	0.25
Tyramine	0.50

by dissolving the evaporated residue from the chloroform-isopropanol extract in iso-amyl alcohol and extract the morphine with boiling diluted sulphuric acid. This purification procedure was used by MARQUIS (1896) for the special purpose of separating amines, socalled "ptomaines" from morphine but tyramine is also extracted quantitatively with 20 ml of boiling 2 N H_2SO_4 from 4 ml of amyl alcohol. A separation of tyramine from morphine may however be performed by paper chromatography mentioned by BROSSI *et al.* (1955) system *A* This procedure is generally used in our Institute, but instead of pH 6.3 as used by Brossi, we generally work at a value of 7.0. A separation can also be performed by chromatography on a plate developed with PARTRIDGE & WESTALL s mixture (1948) see table 2. As well as we can see, it is impossible, however to achieve clear separation between tyramine and morphine by means of descending chromatography on Whatman No 1 paper developed with PARTRIDGE & WESTALL s mixture (1948), a method employed by TRUHAUT & MOAN (1958) for separating alkaloids from biogenic amines ("ptomaines").

Discussion

CURRY (1955) mentions a "phenol-like substance that he assumes to be a precursor of the substance p-hydroxyphenylethanol. As we know that the substance p-hydroxyphenylethanol is formed from tyrosine or tyramine, it seems reasonable to assume that the substance mentioned by Curry is identical with one of these two compounds. As Curry extracts the material with chloroform from an NH_4/NH_3 buffer (on performing a model extraction experiment according to Curry s method, we found the pH of the buffer to be 10.3) tyrosine can be excluded straight away for it cannot be extracted by Curry s method tyramine can, on the other hand, to a certain extent be extracted with chloroform at pH 10.3

There seems every probability that the substance mentioned by CURRY (1955) as a possible precursor of p-hydroxyphenylethanol is tyramine. Tyramine has previously been found in putrefied human intestines, and it has been known by the name of mydine (BRIEGER 1885)

Summary

An impurity frequently occurring in chemical analyses for morphine in autopsy material has been identified as tyramine Methods are described for separating it from morphine

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Absorption of Atropine Anticholinergic Agents as Inhibitors of Absorption from Muscles

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The relative clearance rates of various inert sugars injected into rat muscles were reported in a recent paper (SUND & SCHOU 1964). The rate of sucrose clearance was found to be independent of the concentration of sugar in the injected solutions within a wide range of concentration.

In contrast, the rate of atropine clearance seemed to depend on the concentration. SCHRIFTMAN & KONDRITZER (1957) demonstrated that a solution of lower concentration (2 mg/ml) injected by the i.m. route into guinea pigs has a faster clearance than a more concentrated one (4 mg/ml). This suggests that atropine is retarding its own absorption, possibly by some pharmacological action. Further RAMACHANDRAN & ÅGREN (1963) showed that atropine (about 4–5 mg/ml) added to solutions of di-isopropylphosphofluoridate or inorganic phosphate injected subcutaneously or intramuscularly into rats retarded the absorption of the latter compounds. Also the absorption of sulphacetamide from subcutaneous tissue of mice is retarded by atropine (1 and 10 mg/ml, SCHOU 1963 unpublished results).

In our experiments the rates of clearance of ^{3}H-atropine, injected intramuscularly were determined from solutions containing various concentrations of atropine (0.72–28.6 mM/l). This allows comparison with the rate of clearance of non-polar sugars similar in molecular size. Further an effect on the rate of ^{14}C-sucrose clearance was demonstrated for atropine and compared quantitatively with a similar effect shown by several anticholinergic agents.

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Summary

An impurity frequently occurring in chemical analyses for morphine in autopsy material has been identified as tyramine. Methods are described for separating it from morphine.

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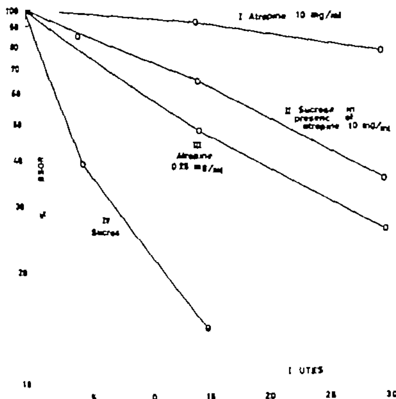


Fig. 1. Clearance curves for atropine sulphate (0.25 and 10.0 mg/ml) and for sucrose with 10.0 mg/ml atropine sulphate. For comparison, a curve for sucrose alone (0.32–0.38 mg/ml, from SUND & SCHOU 1964) is also shown. Concentrations stated on the figure refers only to atropine sulphate.
Abscissa: Time after injection.
Ordinate: Remaining of atropine or sucrose in percent of injected amount (log. scale).

That the relative clearance rate of atropine is to a high degree affected by the concentration of the injected drug was verified in 15 min. experiments with various concentrations of atropine (fig. 2). For comparison, values for sucrose (horizontal line) show that the relative clearance rate of this compound is unaffected by varying the concentration (from SUND & SCHOU 1964). In contrast, a growing decrease in the clearance rate of atropine was demonstrated when the concentration was increased above 0.5 mg/ml up till 10.0 mg/ml. A threshold concentration for this depressing effect of atropine upon its own absorption appears at about 0.5 mg/ml, as the relative clearance is unaltered when the concentration is further lowered to 0.25.

Methods

Male albino rats of a single strain weighing 110–130 g were maintained on a standard laboratory diet with water ad libitum.

The absorption experiments were performed as described in another paper (SUND & SCHOU 1964). Briefly 16 µl of a solution containing the labelled test substance, with sodium chloride added to isotonicity were injected into the exposed *m. extensor quadriceps femoris* of the rats anaesthetized with mebumal i.p. At various times after injection (5 15 and 30 min.) the muscles were removed. After chemical procedure the remaining of the injected compound was determined as radioactivity measured by liquid scintillation technique.

Labelled compounds

Atropine base, generally labelled with tritium, specific activity 470 mC/g, was dissolved with equivalent amounts of weak sulphuric acid.

Sucrose-^{14}C, uniformly labelled, with specific activity of 67 mC/g.

Both radiochemicals were obtained from The Radiochemical Centre, Amersham, England.

Injection solutions

The three types of injection solution were all prepared in water containing 0.9 sodium chloride.

1 Atropine sulphate 0.25–10.0 mg/ml corresponding to 0.72–28 6 mM/l base, containing a standard amount of radioatropine (62.5 µC/ml corresponding to 0.13 mg/ml)

2. Radiosucrose (12.5 µC/ml corresponding to 0.35 mg/ml) with unlabelled atropine sulphate added to 0.125–10 0 mg/ml (0.36–28 6 mM/l).

3 Radiosucrose (12.5 µC/ml, corresponding to 0.35 mg/ml) containing one of various unlabelled anticholinergic drugs at a standard concentration of 14.3 mM/l (equivalent to 5 0 mg atropine sulphate/ml). The anticholinergic compounds were methylscopolamine nitrate (Ph Dan 1948), homatropine bromide (Ph.Dan. 1948), adiphenine chloride (trasentin ®), propantheline bromide (pro-banthine ®) and oxyphenone bromide (antrenyl ®).

Results

Absorption of atropine

The percentage amounts of atropine remaining in the extensor quadriceps femoris muscle were measured 15 and 30 min after injections of 16 µl solutions containing 0.25 and 10 0 mg/ml atropine sulphate (fig. 1) The relative clearance rate is considerably lower from the more concentrated solution. At 30 min. 73 7 ± 1 0 / (n = 4) atropine remained of the 10 0 mg/ml solution, whereas only 25 4 ± 3 8 / (n = 4) was left from the 0 25 mg/ml solution. For comparison a clearance curve for sucrose (0 35 mg/ml) is shown on the figure (from SUND & SCHOU 1964). Sucrose is cleared significantly faster than atropine even from the 0 25 mg/ml solution.

added atropine (10 mg/ml, curve II) is convex upward. Also the line for sucrose with 5 mg/ml atropine (not shown on the figure) is upwardly convex. The same tendency is shown by the clearance curve for 10 mg/ml atropine alone (I), but the curve for 0.25 mg/ml (III) is fairly straight. The latter concentration is below the threshold.

To elucidate further the effect of various concentrations of atropine on the absorption of sucrose, 15 min. experiments were performed with added 0.125, 0.25, 0.50, 1.0 and 2.5 mg/ml atropine sulphate. The results are plotted against the logarithms of the concentration of atropine sulphate in fig. 2. As with the absorption of atropine, a threshold is evident for the retarding effect on the absorption of sucrose 0.125 0.25 and 0.5 mg/ml atropine sulphate do not affect the absorption rate of sucrose. Further increases in concentration of atropine are followed by decreases in the rate of sucrose absorption. The remaining sucrose 15 min. after i.m. injection plotted against the logarithm of the atropine concentration shows a curvilinear regression above this threshold value (fig. 2). The remaining sucrose is considerably lower than that of atropine at similar atropine concentrations. Nevertheless, the threshold for the atropine effect on the absorption and the slope of the dose-response-curves are closely similar for atropine and sucrose.

The retarding effect of atropine on absorption is considered to be a local action. To prove this assumption, a group of rats was injected with unlabelled atropine sulphate (10.0 mg/ml) into the left extensor quadriceps femoris muscle 15 min. before the injection of radiosucrose (0.35 mg/ml) into the right side muscle. After 15 min. 15.5 ± 3.3 / (n = 4) was left of the injected sucrose. In a parallel control group that did not receive atropine, 15.3 ± 2.7 / (n = 5) sucrose remained after 15 min. This experiment excludes a systemic effect of atropine on sucrose absorption.

Absorption of sucrose as affected by other anticholinergics

Several anticholinergic drugs were found to retard the absorption of sucrose. In fig. 3 is shown the remaining amounts of injected sucrose after 15 min., when 14.3 mM/l of atropine, methylscopolamine, homatropine, adiphenine (trasentin ®), propantheline (pro-banthine ®) or oxyphenone (Antrenyl ®) had been added to the injected solutions. Great variation in the retarding effect on absorption is found for equimolar concentrations of the various anticholinergics. Most potent were propantheline and oxyphenone.

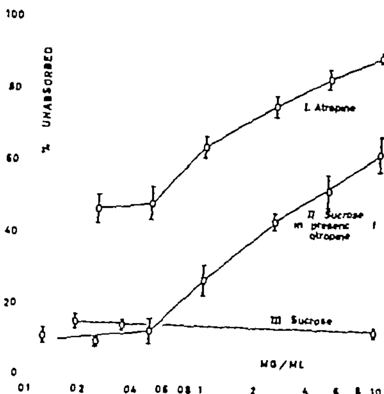


Fig. 2. Dose response curves for the effect of atropine upon absorption of atropine and sucrose (I & II). For comparison, the corresponding curve for sucrose (0.19–9.6 mg/ml, taken from SUND & SCHOU 1964) in complete absence of atropine is also shown (III). Each point represents the mean value of at least four experiments. Vertical bars indicate s.e.m.

Abscissa: Concentration of atropine (I & II) or sucrose (III) (log. scale).
Ordinate: Remaining of atropine or sucrose in percent of injected amount after 15 min.

Absorption of sucrose as influenced by atropine

To find whether or not atropine would affect the absorption rate of an inactive test substance, the clearance of ^{14}C-sucrose (0.35 mg/ml) was measured. When 10.0 mg/ml atropine sulphate were added 83.7 $\pm$ 3.8 (n = 4), 62.7 $\pm$ 5.0 % (n = 6) and 34.4 $\pm$ 2.7 % (n = 4) of the injected ^{14}C sucrose remained at the injection site 5, 15 and 30 min. after the injection, respectively. These results are shown graphically in fig. 1 curve II. Of a control sucrose solution (0.35 mg/ml) only 14.1 ± 1.5 remained after 15 min. (SUND & SCHOU 1964). With 5.0 mg/ml atropine sulphate added to the sucrose the absorption rate of the latter substance decreased but to a less degree than on addition of 10.0 mg/ml. After 15 and 30 min. 52.3 $\pm$ 4.6 % (n = 6) and 25.0 $\pm$ 0.3 (n = 3), respectively of the injected sucrose remained.

The clearance curve for sucrose alone shows a downward convexity in a semilogarithmic plot (fig. 1 IV), whereas the curve for sucrose with

soluble substance such as sucrose (mol. wt. 342) of similar molecular weight. This indicates that the drug is adsorbed or bound to "sites of loss" within the injection zone. The findings of OROSZLAN & MAENGWYN-DAVIES (1962) that atropine reacts with proteins and amino acids is probably of relevance. The lipoproteins of the cell membranes and the amino acids and proteins of the interstitial fluid in that event may constitute the binding sites.

It is also conceivable that atropine is bound within the pores of the capillary wall to a considerable extent, thereby decreasing the effective diffusion area for substances travelling through the water phase, including atropine itself. This explanation for the low basal clearance rate of atropine, however, *appears unlikely from the experiments* in which atropine and sucrose were injected together. As long as the atropine concentration was below the threshold value, the absorption of sucrose was not affected. On the other hand, this mechanism may be of significance for the gradual decline in absorption rate of both substances that is found at the higher atropine concentrations, more and more atropine being bound within the pores.

At higher concentrations, atropine affects its own absorption and the absorption of sucrose in a similar manner. First, the threshold concentration of atropine is the same for both. Secondly, the dose-effect curves (regression of the percentage remaining of either substance on dose of atropine) appear to be parallel. Thirdly, with atropine added at high concentration the clearance curves (the semilogarithmic plot of the percentage residual amounts against time) of both substances show an inverted type of curvature when compared with the slope of the curves obtained for inert sugars (SUND & SCHOU 1964). The same tendency is also shown by atropine curves recorded by SCHRIFTMAN & KONDRITZER (1957).

The similarities outlined above strongly suggest that the graded depression provoked by atropine on the absorption of both atropine and sucrose is due to the same mechanism. Most likely the cause is a decrease in local nutritional capillary flow. Other explanations might be a self depression due to liberation of 5-hydroxytryptamine and histamine (cf. SCHOU 1961) or an effect on the capillary membrane, reducing the size of the individual pores and thereby the water phase area of the membrane, e.g. by the mechanism depicted above. The upward convexity of the clearance curves points to a decrease in hindrance to absorption as time passes.

Several anticholinergic drugs were found to decrease the absorption rate of sucrose. In this connection it may be mentioned that the intestinal absorption of magnesium is greatly accelerated by cholinergic compounds (JENSEN-HOLM 1963). The effect was abolished by atropine, which possibly

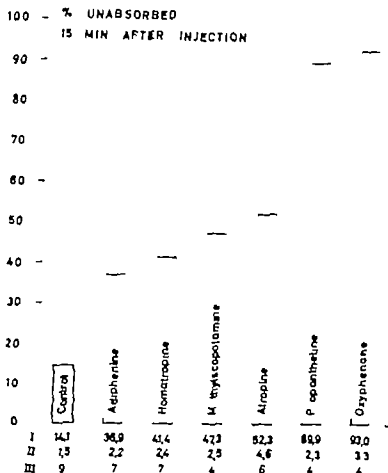


Fig. 3. Comparison of various anticholinergic agents with respect to inhibitory effect upon sucrose absorption. Concentration of anticholinergic agent 14.3 mM/l; of sucrose 0.35 mg/ml. Vertical scale: Remaining of sucrose in percent of injected amount after 15 min. The actual mean residuals (I) are stated below the columns, with corresponding s.e.m. (II) and number of animals (III).

Discussion

The clearance rate (relative clearance) for atropine decreases gradually with increasing concentrations of the drug over a certain threshold level (about 0.5 mg/ml). This is in contrast to the clearance of sucrose, which is independent of concentration over a wide range (SUND & SCHOU 1964).

Even at the threshold concentration, atropine is absorbed at a rate considerably smaller than is to be expected from the molecular dimensions of the drug. Atropine at physiological pH (extracellular 7.4, intracellular 7.0) is completely ionized, having the pK_a value of 9.9 (Ph.Nord.). It can consequently be assumed to be absorbed through the pores of the capillary wall and not through the endothelial lining cells. Nevertheless, the absorption rate for atropine (mol. wt. 289) is less than that for a non polar water

soluble substance such as sucrose (mol. wt. 342) of similar molecular weight. This indicates that the drug is adsorbed or bound to "sites of loss" within the injection zone. The findings of OROSZLAN & MAENGWYN DAVIES (1962) that atropine reacts with proteins and amino acids is probably of relevance. The lipoproteins of the cell membranes and the amino acids and proteins of the interstitial fluid in that event may constitute the binding sites.

It is also conceivable that atropine is bound within the pores of the capillary wall to a considerable extent, thereby decreasing the effective diffusion area for substances travelling through the water phase, including atropine itself. This explanation for the low basal clearance rate of atropine, however, appears unlikely from the experiments in which atropine and sucrose were injected together. As long as the atropine concentration was below the threshold value, the absorption of sucrose was not affected. On the other hand, this mechanism may be of significance for the gradual decline in absorption rate of both substances that is found at the higher atropine concentrations, more and more atropine being bound within the pores.

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decreased the normal absorption. A working hypothesis is that the effect of atropine on the absorption from muscles is due to the capillaries being desensitized to acetylcholine. Commonly however acetylcholine is not considered of importance for maintaining the capillary flow. Thus, it should be of interest to investigate the effect of cholinergic compounds, including inhibitors of acetylcholine esterase, on the absorption of sucrose. Work along this line is in progress.

Summary

Radioatropine (^{3}H) sulphate and radiosucrose (^{14}C) mixed with atropine sulphate or one of 5 other anticholinergic agents was injected into the exposed *m. extensor quadriceps femoris* of anaesthetized rats in a constant volume. Clearance rates of atropine and sucrose were studied by liquid scintillation measurement of residual radioactivity in the muscles. All the anticholinergic compounds depressed absorption of sucrose. The clearance rates of both substances, above a threshold concentration of atropine, decreased with increasing dose of the drug. At doses that did not inhibit the absorption of either substance, the clearance rate of atropine was still lower than is to be expected from molecular size. The self-depression of atropine absorption and the inhibited absorption of sucrose seem to vary in parallel and are thought to be due to the same local pharmacological action of atropine. This probably consists in interference with local blood flow but the exact mechanism is unknown.

Acknowledgement.

This study was supported by a grant from The Foundation for the Advancement of Medical Science, Copenhagen. Mrs. Vivi Halling gave perfect technical assistance. (Adiphenine chloride (trasentin ®) and oxyphenone bromide (antrenyl ®) was kindly presented by Ciba A.G.).

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Acta pharmacol et toxicol 1964 21 347–364

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Effects of Reserpine on the Self-Selected Circadian Rhythm in the Canary

By

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Drugs that cause sleep in the physiological sense can theoretically be divided into two different groups. One consists of drugs that through their action induce sleep when no normal sleep would have occurred, the other group consists of drugs that permit normal sleep to occur at the expected time in spite of factors that would otherwise prevent it. To determine whether a central nervous system depressant belongs to the first group or not, special experimental conditions are needed. This was first clearly realized by HONDELINK (1932).

Self-selected rest and activity in the canary (*Serinus canarius*) have been used in our investigation to evaluate sleep and wakefulness. In the normal self-selected rhythm of this bird there is one period of activity and one of rest and these recur with a period of approximately 24 hours (a circadian rhythm, HALBERG 1959). General surveys of the "biological clock" are given in two recently published books (BÜNNING 1963 and CLOUDSLEY-THOMPSON 1961) and a review by ASCHOFF (1963). The new edition of KLEITMAN's classic (1963) covers the same field for sleep and wakefulness.

With the self-selection method it is possible under controlled conditions to study changes induced in the normal rhythm of activity and rest by various drugs. The effects of reserpine will be presented in this paper. They have been briefly discussed in a previous paper as examples of drug effects on self selected rhythm (WAHLSTRÖM 1964 a).

Methods

Details of the method have been described elsewhere (WAHLSTRÖM 1964 b); they will be only briefly described here.

The canaries, most of them probably young (males, if not otherwise stated), were placed singly in special light-proof wooden cages. The source of light in the cage was a common frosted electric bulb of 75 W unless otherwise stated. In the cages there were

decreased the normal absorption. A working hypothesis is that the effect of atropine on the absorption from muscles is due to the capillaries being desensitized to acetylcholine Commonly however acetylcholine is not considered of importance for maintaining the capillary flow Thus, it should be of interest to investigate the effect of cholinergic compounds, including inhibitors of acetylcholine esterase, on the absorption of sucrose. Work along this line is in progress.

Summary

Radioatropine (^3H) sulphate and radiosucrose (^{14}C) mixed with atropine sulphate or one of 5 other anticholinergic agents was injected into the exposed *m extensor quadriceps femoris* of anaesthetized rats in a constant volume. Clearance rates of atropine and sucrose were studied by liquid scintillation measurement of residual radioactivity in the muscles All the anticholinergic compounds depressed absorption of sucrose. The clearance rates of both substances, above a threshold concentration of atropine, decreased with increasing dose of the drug. At doses that did not inhibit the absorption of either substance, the clearance rate of atropine was still lower than is to be expected from molecular size. The self-depression of atropine absorption and the inhibited absorption of sucrose seem to vary in parallel and are thought to be due to the same local pharmacological action of atropine. This probably consists in interference with local blood flow but the exact mechanism is unknown.

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active substance, and at approximately the same time in the period, distilled water was administered through the stomach tube (1.0 ml/100 g bodyweight). This could not always be done in every circadian period, however. Some estimate of the success can be obtained from fig. 2. When the birds are habituated, administration of distilled water through a stomach tube has no influence on the rhythm (WAHLSTRÖM 1964 b).

In the same bird the interval between two consecutive oral administrations of reserpine was not less than 3 weeks. The commonest interval was a month.

In some experiments reserpine was given as i.m. injection. The concentration of the reserpine used in these cases (serpasil ® Ciba Ltd.) was 1.0 mg/ml. It was administered with a microsyringe into the breast muscle. More than 0.01 ml was seldom needed. No injection of a control solution was performed during the circadian periods before or after the drug injections. There was always at least a month between consecutive administrations.

Chemical analyses.

Two experiments in each of two series were performed. In the first series (experiments a and b, table 3) the birds were kept in a laboratory with alternating light and dark periods (light 8–19 o clock, darkness 19–8 o clock). Experimental and control birds were kept in separate cages. Food and water were always available. Reserpine was given orally in a dose of 0.5 mg/kg at 10 o clock the day before they were killed. The controls received distilled water. Once the light was extinguished (at 19 o clock) on the day the drug was given, there was continuous darkness in the laboratory until after the birds were killed by decapitation between 10 and 10.30 next morning, approximately 24 hours after the reserpine administration. The order in which the birds were killed alternated in the two experiments. Because of this the controls had been awake approximately 20 min. in a dark cage before decapitation in experiment a.

In the second series (experiments c and d, table 3) the intention was to study possible differences in monoamine contents between the waking and the sleeping state. The birds were kept in the cages used in the self-selection experiments, but two in each cage. The light and the dark periods in experiment d were the same as in the first series. In experiment c, however, the clock regulating the light was slow running an approximately 25 hours cycle. This caused the light period to last from 8[30] to 20 o clock. The birds to be killed asleep had no light in the cage on the day they were killed (treated as the controls in the reserpine experiments except for the orally administered water). The other birds had light in the cage from 8–10 o'clock. The birds were beheaded at 10 o clock.

The analyses of noradrenaline were performed on the whole brains by the method of BERTLER, CARLSSON & ROSENGREN (1958) and of dopamine by that of CARLSSON & WALDECK (1958) with slight modifications. The following description includes the main modifications. EDTA 1 mg/ml and ascorbic acid 0.5 mg/kg were added to the perchloric acid used in the extraction procedure. The pH of the extract was adjusted to 6.4 before purification on a column of Dowex 50 W X-4, 400–600 mesh, 5.5 × 35 mm. EDTA (1 mg/ml) were also added to the sodium acetate-acetic acid buffer used in the treatment of the column. Noradrenaline was eluted with 11 ml N hydrochloric acid and dopamine with 11 ml 1.5 N hydrochloric acid in that order. The samples were compared with standard solutions treated in the same way as the eluate. For details see Meyerson (1964).

two perches. The perches were fitted to microswitches, which reacted to a load of 10 g. The microswitch of one of the perches (the night perch) was connected to the lamp of the cage in such a manner that the light was extinguished when the bird used the perch. The other perch (the day perch) was used only to record the activity of the bird when the light was on. The birds thus could choose between darkness and light simply by hopping onto or from the night perch. Each perch actuated one pen on a multichannel recorder. Paper speed was 1.5 inch/h.

Water and food (commercial canary seed mixture) were given *ad libitum*. The animals were, if possible, attended to once every activity period (usually at the same time as distilled water was administered). The efficiency of the night perch was also tested at the same time. Once a week the birds were weighed, and at the same time other foods were given (orange, apple or lettuce, depending on the season and always a piece of hard-boiled egg).

The normal rhythm usually consists of one period of activity (light) and one of rest (darkness) which together give the circadian period. The circadian period is calculated from waking up time to waking up time. Sometimes the activity period is divided into two or more parts by periods on the night perch. Such rest periods when less than 30 min. of the activity period have been disregarded. Periods of 30 min. or more on the night perch during the activity period have been counted as real rest periods and added to the long rest period that followed the activity. This arbitrary limit has been selected because it is unlikely that the birds are really asleep during shorter periods than 30 min. on the night perch. This matter has been discussed in more detail elsewhere (WAHLSTRÖM 1964 b).

Drug experiments

The reserpine (serpidin ® Pharmacia Ltd.) used in the oral experiments was in the form of the commercial solution for intramuscular injection, and the concentration in the undiluted state was 2.5 mg/ml. The solvent consisted of citric acid 2.1 mg, benzyl alcohol 10.0 mg, polyethylene glycol 100.0 mg, ethanol 95 % 10.0 mg, EDTA 0.4 mg and redistilled water to 1.0 ml. Immediately before administration this solution was so diluted with distilled water that the birds always got 1.0 ml/100 g body-weight irrespective of the dose given. The control solution consisted of all the chemicals enumerated above, except reserpine. It was diluted in the same way as to give reserpine at a dose of 2.0 mg/kg. The amount given was 1.0 ml/100 g bodyweight. Benzyl alcohol was given in solutions so diluted with distilled water that the birds recieved 1.0 ml/100 g bodyweight.

All solutions were administered through a stomach tube (a small polyethylene tube fitted to a syringe). After the administration, the birds were observed for approximately 1 min., to ensure that the solution had entered the stomach and remained there (the canary has no crop sac). Each bird had its own stomach tube.

The time at which the drug was given was selected so as to represent three different phases of the bird's circadian period. AM means that the drug was given before, PM that it was given after the middle of the average pre-experimental activity period and N that it was given during the rest period. For the last mentioned administration the bird was aroused, dosed and put back into the cage. Sometimes it was possible to put the bird back onto the night perch directly, but often there was a short activity afterwards, seldom longer than 15 min. Activity periods shorter than 15 min. after drug administration have been disregarded.

In the circadian periods before and after administration of a solution containing an

of fight in the cage after the drug administration, but no recorded activity In some of these direct observations showed the birds to be incapable of using a perch.

The doses of reserpine given in experiments whose results were taken into account were 0.5 mg/kg on 6 occasions to 2 birds, 1 0 mg/kg on 1 occasion, 1.5 mg/kg on 16 occasions to 6 birds and 2.0 mg/kg on 4 occasions to 2 birds, one a female (autopsy) The doses were given at different phases of the circadian period of the birds.

The original record of the effects of reserpine in three birds with different doses is shown in fig. 2. There was AM administrations of the drug on two occasions and PM on one occasion. The effects of the drug were mainly a shortening of activity due to earlier roosting.

In bird no 12 there was a distinct shortening of the activity period after the drug was given. The effect lasted for 2–3 more periods. The shortened activity was expressed as a change in the roosting time. The waking up times were unaffected. Divided activity periods were more frequent after the drug than before.

A similar result was obtained on bird no 16. On day 145, the day after

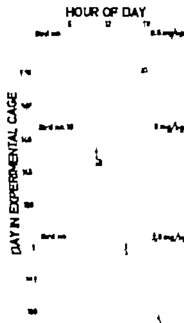


Fig. 2. Effects of reserpine on individual birds. Thin line signifies activity thick line rest. Unmarked arrows indicate administration of distilled water Reserpine was given at the arrows marked xb D A 40 W lamp was used in the cage of bird no. 9 Date of day no 560, 23 Jan. 1962 (bird no 12) day no. 144, 14 Jan. 1961 (bird no. 16) and day no. 184 23 Nov 1960 (bird no. 9).

Results

I *The effects of reserpine on the self selected rhythm*

a. Control experiments, experimental material and individual responses

Since the reserpine solution contains several constituents besides reserpine a number of experiments with the control solution were performed in one bird. If the solution was given undiluted during the rest period of the bird there was a period of activity for some hours afterwards, whereas the bird hopped onto the right perch within 15 min. if distilled water was given. Fig. 1 shows that benzyl alcohol also has this effect. A dose of 110 mg/kg had a very marked effect, 55 mg/kg also showed a slight effect but when the control solution was diluted as if it contained reserpine in a dose of 2 mg/kg there was no effect. The dose of benzyl alcohol in this case was 8 mg/kg.

Reserpine has been administered orally in a single dose on 48 occasions to 14 birds. 21 occasions had to be excluded for various reasons. Of the 14 birds 3 died. The doses given were 3.0 and 2.0 mg/kg. (As a curiosity it may be mentioned that two of the birds drowned themselves!) Reserpine in high doses caused clear incoordination and the birds fell into the water bowl. In 10 instances there were technical errors. In three the recording equipments did not work and in seven the exact dose of reserpine given was uncertain, as some of the volume administered was not retained by the bird. The effects obtained in these cases were those expected from a small dose of reserpine. In 8 tests the birds had long periods

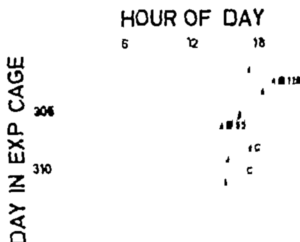


Fig. 1 Effects of benzyl alcohol on the self selected rhythm of bird no. 4 (female). Thin line signifies activity, thick line rest. Unmarked arrows indicate administration of distilled water. B 110 indicates benzyl alcohol 110 mg/kg, B 55 55 mg/kg. C indicates control solution diluted in the same manner as if 2.0 mg/kg reserpine were given (benzyl alcohol 8 mg/kg). A 40 W lamp was used in the cage. Date of day no. 305, 4 Feb. 1961.

of light in the cage after the drug administration, but no recorded activity In some of these direct observations showed the birds to be incapable of using a perch.

The doses of reserpine given in experiments whose results were taken into account were 0.5 mg/kg on 6 occasions to 2 birds, 1 0 mg/kg on 1 occasion, 1.5 mg/kg on 16 occasions to 6 birds and 2.0 mg/kg on 4 occasions to 2 birds, one a female (autopsy) The doses were given at different phases of the circadian period of the birds.

The original record of the effects of reserpine in three birds with different doses is shown in fig. 2. There was AM administrations of the drug on two occasions and PM on one occasion. The effects of the drug were mainly a shortening of activity due to earlier roosting.

In bird no. 12 there was a distinct shortening of the activity period after the drug was given. The effect lasted for 2–3 more periods. The shortened activity was expressed as a change in the roosting time. The waking up times were unaffected. Divided activity periods were more frequent after the drug than before.

A similar result was obtained on bird no 16 On day 145 the day after

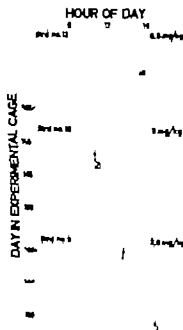


Fig. 2. Effects of reserpine on individual birds. Thin line signifies activity thick line rest. Unmarked arrows indicate administration of distilled water Reserpine was given at the arrows marked with D A 40 W lamp was used in the cage of bird no. 9 Date of day no 340, 23 Jan. 1962 (bird no. 12) day no. 144, 14 Jan. 1961 (bird no. 16) and day no. 184, 23 Nov 1960 (bird no. 9).

the drug, there was little activity Not until day 149–150 had the activity period returned to its pre-experimental length. Waking up on day 145 was retarded With this exception waking up times were unaffected. The lengths of the circadian periods were the same as in the pre-experimental periods. The changes seen in the length of activity were due to changes in the roosting times.

The same pattern is seen also in bird no 9 which had the highest dose of reserpine. The activity was shortened owing to earlier roosting. The pre-experimental length of activity did not return until 7–8 days after the drug administration. Some effect on the waking up times can also be detected, which for some circadian periods after the drug appeared slightly earlier than expected from the pre-experimental period.

b Reserpine and the internal clock governing waking up times.

After reserpine the birds sometimes had no activity in what should have been circadian periods 1 and 2 after the period in which reserpine was given (period no 0) The rest periods were thus longer than 24 or even 48 hours. The course of the waking up times after these long rest periods needs special mention

Fig. 3 shows the waking up times at the beginning of the circadian

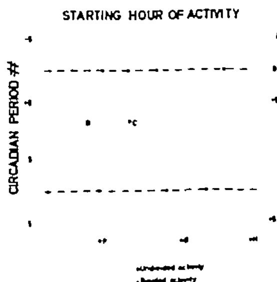


Fig. 3. Effects of oral reserpine on the waking up times in experiments with long rest periods. The drug was administered during circadian period no. 0. Experimental conditions

Experiment	A	B	C	D	E	F	G	H
Dose mg/kg	1.5	1.5	1.5	1.5	0.5	1.5	1.5	1.5
administered	AM	N	PM	N	PM	AM	N	N
Time of waking up in period no −5	3^{56}	2^{12}	5^{41}	21^{07}	1^{14}	16^{30}	3^{04}	23^{13}

periods in those experiments in which there were rest periods longer than 24 hours after oral reserpine. Surprisingly the waking up times after the long rest periods reappeared close to the celestial time to be expected from the pre-experimental series. This was especially clear in experiments A, B, D, G and H. Only the very first waking up showed a small delay in experiments B and H. Thus the waking up times after the long rest periods in most instances seemed to be unaffected by the fact that at least one activity period had disappeared. The "internal clock" governing the waking up times did function without interruption.

Similar results were obtained from some of the intramuscular injections, which produced a loss of at least one activity period (fig. 4). The shape of the curves A and B will be discussed below.

In several of the experiments shown in fig. 3 (A, C, D, G and H) and fig. 4 (A, B, C, D) there were light periods without activity before the long rest periods after the drug. Probably the birds were too sedated to use the perches. These inactive light periods began during what should have been the rest period in circadian period no. 0. Their length was usually 1–4 hours, and they have not been included in fig. 3 and 4.

The duration of the long rest periods in the experiments in fig. 3 were A 25, B 43, C 32, D 25, E 34, F 52, G 43 and H 36 hours and in fig. 4 A 45, B 48, C 29, D 52 and E 41 hours, showing no fixed time-interval between the inactive light period and waking up at the beginning of the first real activity after it. The inactive light periods before the long rest periods thus had no influence on the following waking up time.

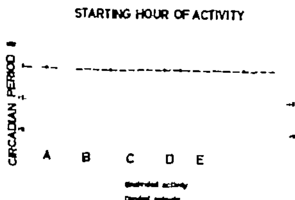


Fig. 4. Effect of intramuscular reserpine on the waking up times in experiments with long rest periods. The drug was administered during circadian period no. 0. Experimental conditions

Experiment	A	B	C	D	E
Dose	0.5	0.5	0.38	0.38	0.5
Administered	N	PM	N	PM	N
waking up in period no. −5	815	2049	1057	312	033

Below is described the effect of reserpine on the different variables of the circadian rhythm. In order to avoid bias the results of all experiments with the same dose given at the same time in the circadian rhythm should, if possible, be averaged. To include the experiments in which long rest periods appeared the fact has been used that the "internal clock regulating waking up times is not stopped by reserpine The average length of the circadian periods was calculated from the time between the waking up beginning the activity in circadian period no 0 to the waking up beginning the activity in circadian period no 3 divided by the number of circadian periods (3) which would have occured if the pre reserpine rhythm had persisted In this manner the results of the experiment could still be used even if all activity had been lost in circadian periods 1 and 2. The mean activity in the two circadian periods after reserpine was similarly evaluated by dividing the total by two Activity in circadian period no 0 was always present and could be estimated separately

A detailed analysis of the length of the circadian periods after reserpine in all the oral experiments is shown in fig. 5 The lengths of the circadian periods are expressed as percentages of the average of the 5 periods just before that one in which reserpine was given. The drug was given in period no 0 Results for this period and the next two (periods 1 and 2) have been averaged (see above) The length of the circadian period was clearly not affected in any experiments except the single one performed with the large dose 2.0 mg/kg given during the night.

In this experiment the first waking up after reserpine was delayed by four hours This affected the average length of circadian periods 0–2. The next four circadian periods were shorter than normal Thus, the waking up times behaved as in experiments A and B (fig. 4), which also involved a high dose, (0 5 mg/kg i m) In these experiments the lengths of the circadian periods did not return to approximately pre-experimental lengths until period 16(A) and 21(B)

Thus moderate doses of reserpine have no effect on the length of the circadian period, but higher doses may have a tendency to shorten it.

c. Reserpine and the length of the activity periods.

Reserpine has a tendency to cause divided activity periods, as mentioned earlier One example was shown by bird no 12, fig. 2. This is further shown in fig. 3 and 4 where the beginning of activity periods that were divided are indicated by triangles.

The effects of reserpine on the lengths of the activity periods are shown in fig. 6, 7 and 8 The effects are expressed as percentages of the average activity in the five circadian periods just before the one in which reserpine

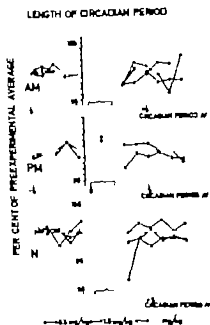


Fig. 5. Effect of reserpine on the length of the circadian periods. The length is given as percentage of the average of the pre-experimental circadian periods. The drug was given orally during circadian period no. 0. Lengths of periods 0–2 have been averaged (see text). In all experiments with 2.0 mg/kg and in one with 1.5 mg/kg in each of the AM and N series 40 W lamp was used in the cage. The N experiment and two AM experiments with 2.0 mg/kg were performed on female

Average length of the pre-experimental circadian periods in hours and the number of experiments are given below

	AM			PM		N		
Dose mg/kg	0.5	1 5	2.0	0.5	1 5	0.5	1.5	2.0
A length hrs	22 9	23.2	24.5	22.7	23.8	22.5	23.5	24.0
n	2	6	3	2	4	2	6	1
Remarks		– 4 from per 5	n = 2 f om per 5					

was given. The drug was given in circadian period no 0. The activity in periods 1 and 2 have been averaged (see part 1b). The periods with light in the cage but no recorded activity which sometimes occurred in the rest period of circadian period no 0, already mentioned in part 1b, have been counted as showing activity

The results of the AM experiments are shown in fig. 6. The amount of activity that had occurred when the drug was given is indicated by the horizontal bars in the lower part of the figure. This corresponds to the

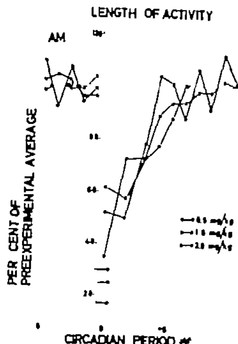


Fig. 6. Effect of reserpine on length of activity period when the drug was given early in the activity period (AM) The length is given as a percentage of the average of the pre-experimental activity periods. The drug was given orally during circadian period no. 0. Lengths of periods 1–2 have been averaged (see text) The bars indicate the average activity already over when the drug was administered. Average pre-experimental activity was, in the experiments with 0.5 mg/kg, 9.5 hrs. 1 5 mg/kg 10.8 hrs. 2.0 mg/kg, 14 0 hrs. See also legend of fig. 5 for further information on the AM experiments.

minimal amount of activity the birds could show in period no 0 The loss of activity due to reserpine is clear and also the gradual return to the pre-experimental length of activity during several circadian periods. There was no clear-cut dose relationship to the amounts of activity lost in the different circadian periods The experiments in the 0.5 and 2.0 mg/kg series were, however few in number

The PM experiments represented in fig. 7 gave essentially the same results. In period no 0 the loss of activity was less because of the late administration of the drug. The N experiments (fig. 8) showed no loss of activity in period no 0 since normal activity had already occurred when the drug was given The single experiment with 2.0 mg/kg showed prolonged activity in period 0 because the bird did not return to the night perch for several hours after the drug was given, whereas in the 0 5 and 1.5 mg/kg series the birds immediately returned to the night perch.

Reserpine thus shortens activity with only a slight effect on the length of the circadian period. Consequently the effects on the rest period were the opposite of those on the activity period and are not shown here.

The effects of reserpine on activity last for several circadian periods. If these losses are summed, the birds lose a considerable amount of activity compared with the pre-experimental period (table 1) The loss of activity is given as parts of the pre-experimental activity period It can be seen

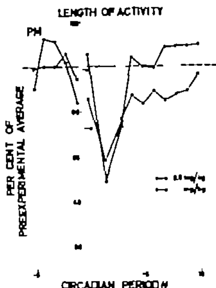


Fig. 7. The effect of reserpine on length of activity period when the drug was given late in the activity period (PM). The length is given as percentage of the average of the pre experimental activity periods. The drug was given orally during circadian period no. 0. Lengths of periods 1 2 have been averaged (see text). The bars indicate the verage activity already over hen the drug was administered. Average pre-experimental activity was, in the experiments with 0.5 mg/kg 9 7 hrs. 1.5 mg/kg 11.0 hrs. See also legend of fig. 5 for further information on the PM experiments.

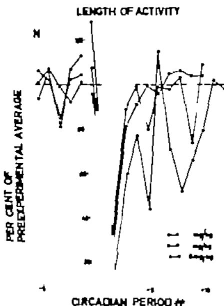


Fig. 8. The effect of reserpine on length of activity period when the drug was given during the rest period (N). The length is given as percentage of the verage of the pre-experimental activity periods. The drug was given orally during circadian period no. 0. Lengths of periods 1 2 hrs been veraged (see text). Average pre-experimental activity was, in the experiments with 0.5 mg/kg, 10.0 hrs. 1.5 mg/kg, 8.5 hrs. 2.0 mg/kg, 11.0 hrs. Average per ...es of the pre-experimental rest period already passed when the drug was given were, ...ments with 0.5 mg/kg, 30.5 %; 1.5 mg/kg, 43 4 % 2 0 mg/kg, 22.1 %. See also legend of fig. 5 for further information on the N experiments.

Table 1

Average loss of activity after oral reserpine (the s.e.m. values are given for the 1.5 mg/kg experiments)

Dose mg/kg	Time	Loss of activity in period (activity periods) 0	n	1 2	n	Total loss of activity within periods (activity periods) 0–2	n	0–4	n	0–6	n	0–8	n
0.5	AM	0.48	2	1.00	2	1.48	2	1.66	2	1.77	2	1.79	2
	PM	−0.06		1.02	2	0.96	2	1.19	2	1.20	2	1.03	2
	N	−0.07	2	1.34	2	1.27	2	1.39	2	1.60	2	1.58	2
	N PM			0.32		0.31		0.20		0.40		0.55	
1.5	AM	0.39 0.11	6	0.85 0.27	6	1.24 ± 0.27	6	1.62 ± 0.54	6	1.92	4	1.96	4
	PM	0.14 0.06	4	0.83 0.27	4	1.34	4	1.85	4	1.60	4	1.88	4
	N	0.04 0.04	6	1.47 ± 0.07	6	0.97 ± 0.22	4	1.33 ± 0.24	4	1.83	6	1.73	6
	N PM			0.64		1.52 ± 0.05	6	1.79 ± 0.13	6	0.23		−0.15	
						0.55		0.46					

that the main loss was suffered within the first 4 circadian periods after the drug at the doses of 0.5 and 1.5 mg/kg and amounted to between 1 and 2 activity periods. It was somewhat higher after 1 5 mg/kg, but the differences were not large compared with the standard error of the mean.

The loss of activity depends on when the drug was given. Experiments performed at AM led to a loss in circadian period 0 which the PM experiments could not produce. This explains the difference between the *AM and the PM values of period no. 0* *During periods 1 and 2 the PM* and AM experiments showed the same loss, but the N experiments gave a higher loss. Considering the duration of the effect (two circadian periods), the small time interval between the drug administrations in the PM and the N experiments cannot explain the difference in loss of activity The difference between losses during period 0–2 in the N and PM experiments with 1.5 mg/kg had a P of 0 02. Taken together with the result in the 0.5 mg/kg experiments it gave a *P* value of less than 0.01 (Student s t)

There are several possible explanations for the difference in the loss of activity between N and PM experiments. It could be caused by different rates of absorption of reserpine from the gastrointestinal tract or differences in elimination due to circadian changes in the function of the liver comparable to the changes in glycogen in the chicken (ELFVIN, PETRÉN & SOLLBERGER 1955). Another possibility is that the cause of the difference lies in the central nervous system. If this were so many interesting questions would arise. The difference found between the effects of reserpine after a N and after a PM administration thus warrants further studies.

2. *Reserpine and birds with irregular rhythms*

Some of the birds have an irregular rhythm. They do not use the night perch at all during long time periods (WAHLSTRÖM 1964b) Reserpine was tried on some of the birds with this irregularity

Nine experiments were performed, the results being shown in table 2. In some a marked regularization was observed (fig. 9).

3. *Analyses of noradrenaline and dopamine in the brain*

The results of the determinations of dopamine and noradrenaline are shown in table 3 After reserpine there was a marked decrease in the amounts of noradrenaline and dopamine compared with the controls. To establish this in the canary was the main aim of these analyses.

Some experiments were also performed to establish whether sleep induced by other methods (moderate prolongation of the dark period) could also accomplish a detectable reduction in the *total amounts of*

Table 1

Average loss of activity after oral reserpine (the s.e.m. values are given for the 1.5 mg/kg experiments)

Dose mg/kg	Time	Loss of activity in period (activity periods) 0	n	1	n	Total loss of activity within periods (activity periods) 0–	n	0–4	n	0–6	n	0–8	n
0 5	AM	0 48	2	1 00		1 48	2	1 66	2	1 77	2	1 79	
	PM	−0 06	2	1.02	2	0 96	2	1 19	2	1.20	2	1 03	2
	N	−0 07	2	1 34	2	1.27	2	1 39	2	1 60	2	1.58	2
	N PM			0 32		0 31		0.20		0.40		0.55	
1 5	AM	0 39 ± 0 11	6	0 85 ± 0 27	6	1 4 ± 0 27	6	1 62 ± 0.54	6	1 92	4	1 96	4
						1 34	4	1 85	4				
	PM	0.14 ± 0.06	4	0 83 0.27	4	0.97 ± 0.22	4	1 33 ± 0.24	4	1 60	4	1 88	4
	N	0.04 ± 0.04	6	1 47 ± 0.07	6	1 52 ± 0.05	6	1 79 ± 0 13	6	1 83	6	1 73	6
	N PM			0 64		0.55		0 46		0.23		−0.15	

Table 3

Amounts of noradrenaline and dopamine in the brains of canaries asleep, wak after reserpine.

Exp	Experimental conditions	Light in the cage before beheading hours	Treatment (orally) 24 hours before beheading	Pooled brains	Number of *females*	Noradrenaline μg/g ±	Dopamine μg/g ± s	Remarks
	Reserpine asleep	0	reserpine 0.5 mg/kg	4	2	0.29	0.98	Asleep
b		0	reserpine 0.5 mg/kg	4	1	0.26	1 16	Asleep
Average		0		4	1 5	0.28	1.07	Asleep
c	No drug wak	2.0		2	0	0.74	1 48	Awak
d		2.2		2	1	0.57	1 62	Awake
Average		2.1		2	0.5	0.66	1.55	Awak
	No drug asleep	0	Water	2	1	0.82	1 73	Awak 20 min.
b		0	Water	2	0	0.52	1 69	Asleep
		0		2	0	0 87	1 63	Asleep
d		0		2	1	0 63	1 62	Asleep
Average		0		2	0.5	0.71 ± 0.16	1 67 ± 0.04	Asleep

Table 2

Oral administration of reserpine to birds with irregular rhythms (long light periods)

Bird no.	Dose mg/kg	Number of experiments successful ¹)	Number of experiments unsuccessful	Remarks
5	2.0		1	40 W lamp
9	2.0	2	1	Uncertain dose 60 W lamp
9	2.0	2		60 W lamp
15	2.0		1	60 W lamp
15	1.5	1		60 W lamp
55	0.5	1		
	Total	6	3	

¹) Regularized rhythm for at least four succeeding circadian periods after the drug administration.

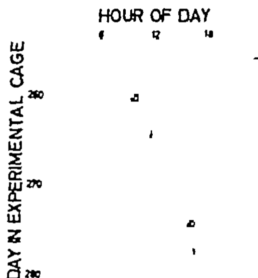


Fig. 9 Effects of reserpine on bird no. 9 with irregular rhythm. Thin line signifies light in the cage, thick line darkness. Unmarked arrows indicate administration of distilled water. Reserpine was given at the arrows marked with a D. On day 261 2.0 mg/kg was given and on day 275 the dose was uncertain (at most 2.0 mg/kg). A 60 W lamp was used in the cage. Date of day 60 7 Feb. 1961

noradrenaline and dopamine in the brains of the canaries. This it clearly did not.

The birds used in the different experiments were rather unhomogenous in sex, age and genetical make-up. The fairly large variation in the amounts of noradrenaline found among the different experiments was probably due to this.

Table 3

Amounts of noradrenaline and dopamine in the brains of canaries asleep wak or after reserpine.

Exp	Experimental conditions	Light in the cage before beheading hours	Treatment (orally) 24 hours before beheading	Pooled brains	Number of females	Noradrenaline μg/g ±	Dopamine μg/g ±	Remarks
	Reserpine	0	reserpine 0.5 mg/kg	4	2	0.29	0.94	Asleep
b	asleep	0	reserpine 0.5 mg/kg	4	1	0.26	1.16	Asleep
Average		0		4	1.5	0.28	1.07	Asleep
	No drug	2.0		2	0	0.74	1.48	Awak
d	wake	2.2		2	1	0.57	1.62	Awake
Average		2.1		2	0.5	0.66	1.55	Awak
	N drug	0	Water	2	1	0.82	1.73	Awake 20 min.
b	asleep	0	W ter	2	0	0.52	1.69	Asleep
		0		2	0	0.87	1.63	Asleep
d		0		2	1	0.63	1.62	Asleep
Average		0		2	0.5	0.71 ± 0.16	1.67 ± 0.04	Asleep

Discussion

Reserpine changed the self selected circadian rhythm in such a manner that the amount of activity was reduced and the amount of rest increased, but the circadian period which is the sum of these two was mainly unaffected Since reserpine is a drug with many pharmacological actions (reviews BEIN 1956 and SCHNEIDER 1957) some possible explanations of the results will be discussed.

Reserpine lowers the body temperature in different species (BEIN 1956) It is possible that this lowering of the temperature was responsible for the loss of activity HOFFMAN (1958) has studied the effects of reserpine on the temperature of pigeons. Reserpine was given in a dose of 2.5 mg/kg subcutaneously and had a marked influence on the cloacal temperature However after 24 hours the effects had passed. This seems to be true of most species (SCHNEIDER 1957) In the canary a lower dose of oral reserpine affected the activity for at least 4 times longer a period than that of the temperature effect in the pigeon Further SCHWARTZBAUM (1955) in experiment on the rat showed that these animals preferred a warm environment after reserpine. The cages of the canaries are warmer with the light on (WAHLSTRÖM 1964b) A lowered temperature, as such, is thus *not* very probable as an explanation of the activity loss.

In many species reserpine exerts a sedative action, which is different from that of the barbiturates (BEIN 1956) The animals are calmer and less aggressive than in the normal state (tranquilized) This effect depends on the action of reserpine on the central nervous system, probably connected with the depletion of some amine The release of serotonin (5-hydroxytryptamine) and catecholamines by reserpine has recently been reviewed by SHORE (1962)

In the early investigations of reserpine there were different opinions about whether sleep was induced in the animals or not BEIN (1953) after studies on dogs cats and rabbits was of the opinion that the animals did sleep more after reserpine PLUMMER *et al* (1954) after experiments mainly on the dog and the monkey concluded that reserpine in contrast to nembutal acts primarily as a tranquilizer and inactivating agent, which may permit but does not cause sleep by a direct hypnotic action Our investigation shows that the canary did indeed choose an environment normally connected with sleep (WAHLSTRÖM 1964b) No direct observations of the birds during the daily rest periods after reserpine were performed, but the birds sat as usual without recorded movements on the night perch. It is thus clear that the birds after administration of reserpine did rest and probably also did sleep much more than usual In the canary reserpine seems to be a true hypnotic drug it induces sleep

It would be astonishing if this effect of reserpine in the canary were not equivalent to the sedation seen in other species and so achieved through an effect on the central nervous system. However the remarkable fact has been established that this effect on the relation between sleep and wakefulness can be accomplished without affecting the biological clock mechanism responsible for the length of the circadian period.

Acknowledgement.

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Summary

If canaries kept in light-proof cages are allowed to select light and darkness in the cage, they normally select one period of light (activity) and one of darkness (rest). These two periods make up the circadian period of about 24 hours, calculated from the beginning of one light period to the beginning of the next.

After oral reserpine (0.5–1.5 mg/kg) the activity periods were shorter and the rest periods longer Usually within 4 circadian periods the duration of activity had returned to the pre-experimental level. The total loss of activity during this time amounted to 1–2 pre-experimental activity periods. The loss of activity was usually accomplished through a change in the roosting times, that is, "going to sleep earlier" The start of the activity periods and, consequently the length of the circadian periods were largely uninfluenced. In some experiments one or two whole activity periods disappeared. Even then the ensuing activity periods tended to begin at the celestial time expected from the pre-experimental periods. Reserpine thus could cause losses of whole activity periods without disturbing the "internal clock" that initiates the activity periods.

Between the experiments in which reserpine was given late in the activity period or alternatively during the rest period a difference in the relative loss in activity was found. Administration of drug during the rest period produced the greater loss in activity

There was a decreased amount of noradrenaline and dopamine in the brains of the canaries 24 hours after reserpine administration.

In the canary reserpine causes the bird to roost earlier and thus seems to be a true hypnotic drug.

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From the Department of Pharmacology University of Copenhagen
(Professor Knud O Møller M.D.)

Effects of Adrenalectomy on Connective Tissue Electrolytes in Mice

By

Hans Langgård

(Received July 24, 1964)

The arrangement of loose connective tissue as a multi-phase system with a large fraction of the tissue water contained in submicroscopic vacuoles (EILERS & LABOUT 1954 GERSH & CATCHPOLE 1960) or attached to hydrophilic side groups on the collagen molecule (FRASER & MACRAE 1959), as well as the presence in the tissue of charged macromolecules as mixed cation-anion exchange-resins (USSING *et al.* 1960), indicates that connective tissue may serve as a "reservoir" for water and electrolytes.

Experiments on mice (HVIDBERG, JENSEN-HOLM & LANGGÅRD 1963 LANGGÅRD, JENSEN-HOLM & HVIDBERG 1963 HVIDBERG, SZPORNY & LANGGÅRD 1964 LANGGÅRD HVIDBERG & SZPORNY 1964) have further demonstrated that important changes in total water and electrolyte content do occur under various conditions, in the connective tissue without similar changes in the blood plasma. This paper deals as a part of these investigations with the effects of adrenalectomy on the water and electrolyte composition of skin and blood plasma in mice.

Methods

White male mice of a single strain, weighing from 23 to 27 g, were used.

Experimental groups.

A 1) Adrenalectomy } killed 24 hours later
2) Sham-operation } killed 24 hours later
3) No operation } killed 24 hours later

After the operation animals in group A were given water ad libitum but no food.

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White male mice of a single strain, weighing from 23 to 27 g, were used.

Experimental groups.

A 1) Adrenalectomy
2) Sham-operation
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After the operation animals in group A were given water ad libitum but no food.

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Table I

Concentrations of sodium, chloride and potass m in plasma nd tissue water (mEq per litre).

		)	N mEq/l plasma	N mEq/l tissue water	Cl mEq/l plasma	Cl mEq/l tissue water	K mEq/l plasma	K mEq/l tissue water
	Adrenalectomy 1 day	12	143 ± 1 4)	73 ± 2.4)	100 ± 1 1	100 ± 2.4	4 2 ± 0.04)	68 ± 3.9
	Sham-operation	3	152 ± 1 7	91 ± 1 4	100 ± 2.0	101 ± 4.3	3 5 ± 0.14	64 ± 3.6
	Control	3	150 ± 0.0	94 ± 4.3	105 ± 1.5	103 ± 1.8	3.3 ± 0.08	70 ± 0.3
	Adrenalectomy 7 days	10	149 ± 0.8	78 ± 2.9)	105 ± 1.8	91 ± 2.3	3.7 ± 0.11	66 ± 2.8
	Sham-operation	3	151 ± 2.5	95 ± 4 4	107 ± 1.8	97 ± 3.2	3 2 ± 0.23	71 ± 6.9
	Control	3	150 ± 2.6	97 ± 2 6	106 ± 1 7	97 ± 5.2	3 3 ± 0.13	75 ± 4 7
Pretreated with oestradiol	Adrenalectomy 1 day	7	146 ± 0 9	107 ± 6.8)	103 ± 1.3	106 ± 4.1	3 6 ± 0.11	45 ± 3.2+)
	Sham-operation	2	151 ± 3.0	128 ± 1.0	104 ± 1.0	113 ± 2.5	3 3 ± 0.20	33 ± 0.5
	Control	4	150 ± 2.3	125 ± 1 7	106 ± 2.3	111 ± 4.2	3 4 ± 0.30	31 ± 3.9

The values re means ± standard errors of the means.
) = number of animals.
) Significantly different from the control group and sham-operated gro p t p < 0 01

B 1) Adrenalectomy
2) Sham-operation
3) No operation
} killed 7 days later

These animals had free access to a standard laboratory diet. In addition they were given 0.9 % NaCl in the drinking water

C 1) Adrenalectomy
2) Sham-operation
3) No operation
} pretreated with oestradiol for 6 days; killed 24 hours later

These animals received water ad libitum but no food (same as group A).

Experimental procedures

The mice in group C were treated with s.c. oestradiol monobenzoate, 10 μg in 0.1 ml of arachis oil, injected 6 and 4 days before the operation.

Bilateral adrenalectomy was performed dorsally with the animal under light fluothane ® (halothane B.P.) anaesthesia. The incision of the skin was made ½ cm to the left of the midline. The sham-operated animals underwent the same procedure, except that the adrenal glands were not removed.

Twenty-four hours or 7 days after the operation the animal was stunned by a blow on the neck, the right carotid artery was cut, and 500 μl of blood were sampled in a Carlsberg constriction pipette treated with heparin. Subsequently the animal was beheaded and bled. The back skin was depilated by a close shave and then by applying a barium sulphide depilatory. A semilunar particular area of the skin, 5.5 cm^2 was marked out and excised with the underlying subcutaneous tissue from the right side of the back. The skin samples were weighed, dried, defatted and analysed.

Hydroxyproline, hexosamine, sodium, chloride and potassium determinations on skin samples and sodium, chloride and potassium determinations on plasma samples were carried out as described by LANGGÅRD, JENSEN-HOLM & HVIDBERG (1963).

Results

In table 1 are shown the concentrations of sodium, potassium and chloride in plasma and tissue water (mEq per litre plasma or total tissue water).

Twenty four hours after adrenalectomy (group A) a slight decrease in plasma sodium and a slight increase in plasma potassium were observed ($p < 0.01$). The concentration of sodium in the tissue water was markedly reduced.

Seven days after adrenalectomy (group B) significant changes in plasma electrolyte concentrations were no longer present, but the sodium concentration was still significantly reduced in the tissue water.

Animals pretreated with oestradiol (group C) showed no significant changes in plasma electrolyte concentrations 24 hours after adrenalectomy. In the tissue water, however, the sodium concentration was reduced and the potassium concentration raised.

Table 1

Concentrations of sodium, chloride and potassium in plasma and tissue water (mEq per lit)

		n)	Na mEq/l plasma	Na mEq/l tissue water	Cl mEq/l plasma	Cl mEq/l tissue water	K mEq/l plasma	K mEq/l tissue water
	Adrenalectomy 1 day	12	143 ± 1.4)	73 ± 2.4)	100 ± 1.1	100 ± 2.4	4.2 ± 0.04)	68 ± 3.9
	Sham-operation	3	152 ± 1.7	91 ± 1.4	100 ± 2.0	101 ± 4.3	3.5 ± 0.14	64 ± 3.6
	C ntrol	3	150 ± 0.0	94 ± 4.3	105 ± 1.5	103 ± 1.8	3.3 ± 0.08	70 ± 0.3
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	Sham-operation	3	151 ± 2.5	95 ± 4.4	107 ± 1.8	97 ± 3.2	3.2 ± 0.23	71 ± 6.9
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The values means ± standard errors of the means.
) n = number of animals.
) Significantly different from the control group and sham-operated group t p < 0.01

B 1) Adrenalectomy
2) Sham-operation } killed 7 days later
3) No operation

These animals had free access to a standard laboratory diet. In addition they were given 0.9 % NaCl in the drinking water

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Twenty four hours after adrenalectomy (group A) a slight decrease in plasma sodium and a slight increase in plasma potassium were observed ($p < 0.01$) The concentration of sodium in the tissue water was markedly reduced

Seven days after adrenalectomy (group B) significant changes in plasma electrolyte concentrations were no longer present, but the sodium concentration was still significantly reduced in the tissue water

Animals pretreated with oestradiol (group C) showed no significant changes in plasma electrolyte concentrations 24 hours after adrenalectomy In the tissue water however the sodium concentration was reduced and the potassium concentration raised

nounced in oestradiol-treated animals, therefore supports the idea that normal plasma electrolyte concentrations are maintained by tissue electrolytes.

COLE (1952), investigating the electrolyte composition of rat's skin after adrenalectomy ascribed most of the changes to a shift of water from the extracellular to the intracellular space. GAUDINO (1954) in experiments on dogs also found a decrease in the inulin space and an increase in the intracellular space after adrenalectomy. DULCE, GÜNTHER & SCHÜTTE (1958) and GÜNTHER, DULCE & SCHÜTTE (1961) on the other hand, found that the water content of the rat's skin after adrenalectomy increased more than could be accounted for by the intracellular uptake, while the "sodium space" (i.e. the part of the extracellular space that would contain all the sodium at the same concentration as in plasma) remained unchanged. They assumed that there was a swelling of collagen fibres with an increase in the amount of "non-solvent water" (i.e. water that does not dissolve electrolytes).

The results of our study indicate that a shift of water from the extracellular to the intracellular space is of little importance for electrolyte changes after adrenalectomy. If the reduction in tissue sodium observed 24 hours after adrenalectomy (table 1) were due to a reduction in extracellular fluid volume at the same time as the total amount of tissue water is significantly raised (table 2), this would imply an enormous increase in intracellular space. The amount of potassium was, however, unchanged. A similar statement can be made about the findings 7 days after adrenalectomy (group B).

In considering the changes that occur in the electrolyte composition of the skin after adrenalectomy, emphasis should be put to the electrolyte-depositing function of connective tissue. The degree of polymerisation of the hyaluronic acid may change after adrenalectomy thereby affecting the water as well as the electrolyte-binding capacity of the tissue. (KULONEN 1952, LANGGÅRD, HVIDBERG & SZPORNY 1964). Changes also may occur in the amounts of collagen fibres and of the water attached to them (GÜNTHER, DULCE & SCHÜTTE 1961).

Summary

The effects of adrenalectomy on the water and electrolyte composition of connective tissue and plasma have been studied in mice.

The findings indicate that the extracellular space may serve as "reservoir" for electrolytes where potassium can be deposited and whence sodium can be mobilized. Normal plasma values may eventually be maintained.

Table 2

Relative amounts (g or mg per 100 g dry fat-free tissue) of water hexosamine and hydroxyproline in the skin.

		n)	Water g/100 g	Hexosamine mg/100 g	Hydroxypro-line g/100 g
	Adrenalectomy 1 day	12	315 ± 5 9)	562 ± 15 4	7 8 ± 0 49
	Sham-operation	3	277 ± 5 4	553 ± 13 0	8.9 ± 0.00
	Control	3	294 ± 8 8	581 ± 22.8	8.9 ± 0.35
	Adrenalectomy 7 days	10	299 ± 7 8	500 ± 13.8	7 7 ± 0.22
	Sham-operation	3	276 ± 12.7	492 ± 8.7	8.4 ± 0.23
	Control	3	286 ± 7 6	482 ± 9.3	7 8 ± 0.59
Pretreated with oestradiol	Adrenalectomy 1 day	7	527 ± 34 5	1049 ± 51 9	6.9 ± 0.26
	Sham-operation	2	528 ± 57 5	1132 ± 65 0	7.3 ± 0.95
	Control	4	562 ± 33 1	1070 ± 40.5	7 3 ± 0 40

All values are means ± standard errors of the means.
) n = number of animals.
) Significantly different from the sham-operated group at $p < 0\ 01$

Table 2 summarizes the relative amounts (g or mg per 100 g dry fat free tissue) of water hexosamine and hydroxyproline in the different groups. The only significant finding was an increase in the water contents in the skin of adrenalectomized mice 24 hours after the operation, compared with those of the sham-operated animals ($p < 0\ 01$)

Discussion

The results of our study indicate the occurrence of changes in the electrolyte composition of the skin after adrenalectomy That these changes were not simply reflections of the composition of the extracellular fluid is indicated by the plasma analyses

Changes in plasma sodium and potassium were modest 24 hours after adrenalectomy and statistically insignificant 7 days later In both groups, however low concentrations of sodium were found in the tissue water It is tempting to interprete this in terms of compensation of plasma electrolytes by tissue electrolytes, an idea substantiated by the findings in mice pretreated with oestradiol.

Treatment of this particular strain of mice with oestradiol causes approximately 100 / increase in the acid mucopolysaccharides of the skin (cf HVIDBERG SZPORNY & LANGGÅRD 1964) Previous studies have indicated that the electrolyte depositing capacity of connective tissue is thereby increased (LANGGÅRD JENSEN HOLM & HVIDBERG 1963 LANGGÅRD 1964) The fact that plasma electrolyte concentrations in the study recorded here were less affected by adrenalectomy in oestradiol treated mice than in untreated mice, whereas tissue changes were more pro-

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Effects of Ascorbic Acid on Electrolyte Composition of the Skin of Mice

By

Hans Langgård

(Received July 24, 1964)

In a series of publications from this laboratory reports on the electrolyte composition of connective tissue have been made. The results have indicated that connective tissue, in a variety of circumstances, may serve as a "reservoir" for electrolytes (HVIDBERG, JENSEN-HOLM & LANGGÅRD 1963 LANGGÅRD, JENSEN-HOLM & HVIDBERG 1963 HVIDBERG SZPORNY & LANGGÅRD 1964 LANGGÅRD HVIDBERG & SZPORNY 1964a LANGGÅRD HVIDBERG & SZPORNY 1964b LANGGÅRD 1964a LANGGÅRD 1964b).

As a part of these investigations the effects of ascorbic acid on non-scorbutic animals have been examined. Ascorbic acid has been chosen because the *ascorbate ion is an example of an anion that can be bound to* macromolecules of connective tissue, thereby affecting the distribution of all other ions (ENGEL *et al* 1961)

Methods

White male mice of a single strain, weighing from 22 to 28 g, were used. They were maintained on a standard laboratory diet with water ad libitum.

Experimental groups.

1. *Ascorbic acid treated* Intraperitoneal injections of 25 mg ascorbic acid (1 g/kg) in 0.5 ml of 0.9 % NaCl were given once a day for 3 days.
2. *Control group* (control on group 1) Same as group 1 but without ascorbic acid in the injection fluid.
3. *Oestradiol + ascorbic acid treated* Oestradiol monobenzoate, 10 µg in 0.1 ml of arachis oil were injected subcutaneously 7 and 5 days before the experiment. Ascorbic acid 25 mg in 0.5 ml of 0.9 % NaCl was injected intraperitoneally on each of the last 3 days before the experiment. In some animals the same dose of ascorbic acid was injected once a day for 7 days.
4. *Oestradiol treated* (control on group 3) Same as group 3, but without ascorbic acid in the injection fluid.

Acknowledgements

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(Received July 24, 1964)

In a series of publications from this laboratory reports on the electrolyte composition of connective tissue have been made. The results have indicated that connective tissue, in a variety of circumstances, may serve as a "reservoir" for electrolytes (Hvidberg, Jensen-Holm & Langgård 1963, Langgård, Jensen-Holm & Hvidberg 1963, Hvidberg, Szporny & Langgård 1964, Langgård, Hvidberg & Szporny 1964a, Langgård, Hvidberg & Szporny 1964b, Langgård 1964a, Langgård 1964b).

As a part of these investigations the effects of ascorbic acid on non-scorbutic animals have been examined. Ascorbic acid has been chosen because the ascorbate ion is an example of an anion that can be bound to macromolecules of connective tissue, thereby affecting the distribution of all other ions (Engel *et al.* 1961).

Methods

White male mice of a single strain, weighing from 22 to 28 g, were used. They were maintained on a standard laboratory diet with water ad libitum.

Experimental groups.

1. *Ascorbic acid treated:* Intraperitoneal injections of 25 mg ascorbic acid (1 g/kg) in 0.5 ml of 0.9 % NaCl were given once a day for 3 days.
2. *Control group* (control on group 1): Same as group 1, but without ascorbic acid in the injection fluid.
3. *Oestradiol + ascorbic acid treated:* Oestradiol monobenzoate, 10 μg in 0.1 ml of arachis oil were injected subcutaneously 7 and 5 days before the experiment. Ascorbic acid 25 mg in 0.5 ml of 0.9 % NaCl was injected intraperitoneally on each of the last 3 days before the experiment. In some animals the same dose of ascorbic acid was injected once a day for 7 days.
4. *Oestradiol treated* (control on group 3): Same as group 3, but without ascorbic acid in the injection fluid.

Experimental procedures

The animals were killed and bled 1 hour after the last injection of ascorbic acid. In a representative number of animals from each group 700–800 μl of blood were drawn from the inferior vena cava, with the animal under light ether anaesthesia. Subsequently the animal was beheaded and bled. The back skin was depilated closely with an electric clipper and then by application of a barium sulphide depilatory Of the back skin 5 to 6 cm² with underlying subcutaneous tissue, were excised and weighed on a torsion balance. In 10 mice from group 1 and 10 mice from group 2 a particular area was marked out and excised. The skin samples were freeze-dried to constant weight defatted by repeated treatments with ethyl ether and light petroleum to constant weight and subsequently analysed.

Hexosamine, hydroxyproline, sodium potassium and chloride determinations on skin samples, sodium, potassium and chloride determinations on plasma samples and pH determinations on whole blood were carried out as described by LANGGÅRD JENSEN-HOLM & HVIDBERG (1963). Corrected bicarbonate values (=standard bicarbonate) were calculated with the aid of the Astrup normogram, as modified by SIGGAARD-ANDERSEN (1963)

Results

The values are expressed in mEq mg or g/100 g dry fat free tissue or mEq/litre tissue water Plasma electrolyte concentrations are expressed in mEq/litre plasma All values are given with the standard error of the mean. Statistical comparisons are based on the t test.

Table 1 shows the changes in corrected bicarbonate of plasma induced by treatment with ascorbic acid The figures indicate that mild degrees of non respiratory acidosis were produced in normal and oestradiol treated mice The figures in parenthesis are from a previous study in which similar degrees of acidosis were produced by intraperitoneal injections of

Table 1

Corrected bicarbonate of plasma. Figures in parenthesis from a previous study in which isotonic NH_4Cl was injected instead of ascorbic acid, leading to similar degrees of acidosis.

	n	Corrected bicarbonate of plasma in meq/l mean s.e.m	(mean s.e.m.)
Control	9	16.8 ± 0.45	(15 7 ±0.39))
Ascorbic acid – treated	9	14 1) ±0.37	(12.6) ± 0.32)
Oestradiol – treated	9	16.3 ± 0.23	(14 9 ± 0.31)
Oestradiol + ascorbic acid – treated	9	13 7*) ±0 34	(12.0*) ± 0 45)

) Significantly different from the control group at $p < 0.001$
*) Significantly different from the oestradiol-treated group at $p < 0.001$
) From a previous study (Langgård, Jensen-Holm & Hvidberg 1963).

isotonic ammonium chloride in the same procedural circumstances as in the study being reported here (LANGGÅRD, JENSEN-HOLM & HVIDBERG 1963).

The concentrations of sodium, potassium and chloride in plasma are shown in table 2. A significant fall in plasma chloride ($p < 0.01$) was observed after treatment with ascorbic acid. Similar degrees of acidosis, induced by ammonium chloride, caused the reverse changes, as demonstrated by the figures in parenthesis.

The figures in table 3 illustrate that the amount of fat-free solids per unit area skin surface was unaffected by treatment with ascorbic acid.

The skin compositions of ascorbic acid treated and control mice are

Table 2

Concentration of electrolytes in plasma. Chloride values in parenthesis from previous study in which similar degrees of acidosis had been produced by i.p. injection of isotonic NH_4Cl.

	n	Sodium meq/l	Chloride meq/l		Potassium meq/l
		mean s.e.m.	mean s.e.m.	(mean s.e.m.)	mean s.e.m.
Control	13	147 ± 0.5	109 ± 1.2	(105 ± 1.2)*)	5.3 ± 0.16
Ascorbic acid – treated	14	145 ± 1.1	103 ± 1.5)	(111 ± 1.3)	5.1 ± 0.14
Oestradiol treated	17	146 ± 0.6	108 ± 1.0	(109 ± 1.0)	5.0 ± 0.19
Oestradiol + ascorbic acid-treated	13	148 ± 0.5	100 ± 1.4*)	(107 ± 2.3)	4.9 ± 0.14

*) Significantly different from the control group at $p < 0.01$

) Significantly different from the oestradiol treated group at $p < 0.001$

*) From previous study (Langgård, Jensen-Holm & Hvidberg 1963).

Table 3

Total contents of water, fat and fat-free solids in skin samples of equal surface area from mice treated with ascorbic acid and from control animals.

		Total water (mg) mean s.e.m.	Total fat (mg) mean range	Total fat-free solids (mg) mean s.e.m.
Ascorbic acid treated	10	168 ± 7.0	51 (13 – 97)	53 ± 1.8
Control	10	168 ± 7.1	58 (20 – 118)	51 ± 1.9

shown in table 4. A significant increase in the hexosamine ($p < 0.01$) and a significant decrease in the hydroxyproline contents ($p < 0.001$) were observed in animals treated with ascorbic acid.

The effects of treatment with ascorbic acid on the skin compositions of oestradiol treated animals are shown in tables 5 and 6. Treatment with ascorbic acid for 7 days (table 6) caused a significant increase in tissue sodium ($p < 0.001$) and a decrease in tissue chloride. In animals treated with ascorbic acid for 3 days only (table 5) apparently similar but statistically non significant changes were recorded.

Discussion

It must be emphasised that this study was not meant to be as one of the specific action of ascorbic acid on collagen production, i.e. of the 'anti-scorbutic factor'. The experiments were therefore carried out in mice, which cannot be made scorbutic.

The purpose of the study was to examine *in vivo* the changes, if any, in the electrolyte composition of connective tissue induced by large dosis of an organic anion that can be bound to polylectrolytes in the connective tissue.

That ascorbic acid is extensively bound in the skin is indicated by analytical values (ABT, VON SCHUCKING & ROE 1959). Tissue levels may exceed blood levels by factors of 10 or more, indicating that over 90 % of the tissue anions are present in a bound form.

Chemically ascorbic acid is a strong acid and will produce systemic acidosis when injected in large doses. Therefore, to be able to distinguish between the specific effects, if any, of the ascorbate ion and non specific effects of the acidification, an intimate knowledge of the effects of simple pH-changes on the composition of connective tissue is necessary.

In a previous study LANGGÅRD, JENSEN, HOLM & HVIDBERG (1963) examined the effects of physiological pH-changes on the electrolyte composition of plasma and connective tissue. In the same strain of mice as used in the study reported here, and in the same experimental circumstances, the same changes in corrected bicarbonate of plasma were produced by intraperitoneal injection of the same amounts of H equivalents (3 injections, 0.14 mEq each) in the form of isotonic ammonium chloride. This treatment produced the same changes in the corrected bicarbonate of plasma as were produced by treatment with ascorbic acid in the study reported here (table 1).

It is, however, noteworthy that, though acidosis produced by ammonium chloride caused the expected increase in plasma chloride concentration*), a significant fall in plasma chloride was observed after treatment

*) During non-respiratory acidosis, any fall in the base buffer must be compensated by a rise in the non-buffer anions, e. g. Cl, or a fall in cations, cf. SIGGÅRD-ANDERSEN 1963.

Table 4

Relative amounts of water hexosamine, hydroxyproline, sodium, chloride and potassium in skin of mice treated with ascorbic acid and of control animals.

		Water g/100 g) mean s.e.m.	Hexosamine mg/100 g*) mean s.e.m.	Hydroxyproline g/100 g*) mean s.e.m.	Sodium meq/100 g) mean s.e.m.	Chloride meq/100 g) mean s.e.m.	Potassium meq/100 g) mean s.e.m.
Ascorbic acid treated	24	296 ± 4.7	522 ± 9.2+)	5.40 ± 0.184++)	23.9 ± 0.46	25.6 ± 0.32	23.8 ± 0.78
Control	22	300 ± 7.1	487 ± 9.8	6.16 ± 0.123	25.3 ± 0.52	26.7 ± 0.47	22.6 ± 0.63

) Significantly different from the control group at $p < 0.01$
") Significantly different from the control group at $p < 0.001$
) Dry fat-free tissue.

Table 5

Amounts of water hexosamine, hydroxyproline and electrolytes in skin of mice treated with oestradiol monobenzoate for 7 days and ascorbic acid for three days or with oestradiol monobenzoate alone.

	n	Water g/100 g)	Hexosamine mg/100 g)	Hydroxyproline g/100 g)	Na meq/100 g)	Na meq/l)	Cl meq/100 g)	Cl meq/l)	K meq/100 g)
Oestradiol for 7 days + Ascorbic acid for 3 days	10	544 ± 25 6	950 ± 46.7	4 64 ± 0 150	68 4 ± 4.16	121 ± 3 7	54 2 ± 3.30	99 ± 2.5	23 7 ± 1 05
Oestradiol	10	534 ± 24 0	903 ± 65.5	4 50 ± 0.253	59 9 ± 4 45	111 ± 4 1	53.9 ± 2.95	101 ± 2.1	26.7 ± 1 68

The values are ± standard error of the mean.
) Fat free solids.
) Tissue water

Table 6

Amounts of fat-free solids, water hexosamine, hydroxyproline and electrolytes in skin of mice treated with oestradiol monobenzoate and ascorbic acid for 7 days or with oestradiol monobenzoate alone.

		T tal fat-free solids mg	Water g/100 g)	Hexosamine mg/100 g)	Hydroxy-proline g/100 g)	N		Cl		K
						meq/100 g)	meq/l)	meq/100 g)	meq/l)	meq/100 g)
Oestradiol + ascorbic acid for 7 days	6	79 ± 3 2	657 ± 25 0	1264 ± 68.3	5 43 ± 0.198	92.6)±3.16	141)±3.75	63.6 ± 3 75	97.0 ± 3.51	21 4 ± 0.62
Oestradiol	6	80 ± 1 9	618 ± 16.7	1329 ± 32.8	4.95 ± 0.096	75.5 ± 3.00	122 ± 3 37	66.7 ± 3.37	107 7 ± 2.39	23.6 ± 0 70

The values are means ± standard erro of the mean.
) F t-free solids
) Tissue water
) Significantly different from the oestradiol treated group t $p < 0.001$

with ascorbic acid (table 2) This may have been due to an excretion of Cl^- in the urine. When ascorbate ions are bound in the tissue, hydrogen ions may be excreted together with Cl^- Another possibility is that the ascorbate ions simply compete with inorganic anions of the plasma (e. g. Cl^+)

As to changes in the composition of the skin, the question of a stable basis of calculation is of importance. The values in table 3 indicate that the amount of fat free solids per unit area skin surface was unaffected by treatment with ascorbic acid. The principle of expressing the contents of the various constituents of the tissue in relation to 100 g of the dry fat free tissue could therefore be applied

Treatment with ascorbic acid raised the amount of hexosamine and reduced the amount of hydroxyproline in the skin of non scorbutic animals (table 4) PERSSON (1953) and JORGENSEN (1962) found an increase of hexosamine in the skin of scorbutic guinea pigs. Administration of ascorbic acid caused a rapid change of the values towards normal Others (e.g. CHEN & POSTLETWAIT 1961) have not found this the whole problem of the effects of ascorbic acid on the acid mucopolysaccharides of the connective tissue is far from having been clarified. It is however generally agreed that absence of ascorbic acid leads to a severe inhibition of formation of collagen fibres (GOULD 1958 CHEN & POSTLETWAIT 1961 JORGENSEN 1962) Effects of ascorbic acid on collagen of normal connective tissue have, as far as we know not been reported. The apparent discrepancy between the results of the studies cited above and of our study may therefore not exist, since comparison between the action of ascorbic acid in scorbutic and non-scorbutic animals may have no meaning.

ROBERTSON (1961) found that not only collagen formation but also the break-down of collagen depends on the presense of ascorbic acid. This observation may indicate a parallel with our results Ascorbic acid may be present in mouse skin at the concentration optimal for collagen synthesis. A further supply of ascorbic acid would therefore have no influence on collagen formation but could accelerate the break down of collagen

The most significant changes in the electrolyte composition of the skin are seen in the oestradiol treated animals. The treatment of this particular strain of mice causes an approximate 100 % increase in the amount of acid mucopolysaccharides in the skin The electrolyte binding capacity of the skin is thereby considerably increased (HVIDBERG, SZPORNY & LANGGÅRD 1964). The decreased tissue chloride of these animals was probably the result of a competition between ascorbate ions and chloride ions bound to hyaluronic acid (LANGGÅRD 1964a) The deposition of

sodium after treatment with ascorbic acid seems of special interest, in view of the fact that acidifying treatment with ammonium chloride resulted in a considerable mobilization of sodium from the tissues (LANGGÅRD, JENSEN-HOLM & HVIDBERG 1963) Apparently ascorbic acid has a specific effect on connective tissue ground substance this prevents or over compensates for the non-specific effects of the acidifying treatment. The binding of the ascorbate ions itself may affect the cation binding. The binding of anions to insoluble colloids is generally accompanied by changes in the binding of hydrogen ions (ENGEL & JOSEPH 1960 ENGEL et al. 1961). As a result, the distribution of all anions and cations is affected through modifications of the colloidal charge. Ascorbic acid may further have a direct effect on hyaluronic acid. Although the hexosamine content is unchanged (tables 5 and 6), the hexosamine content in the group treated with ascorbic acid may represent a more highly polymerized hyaluronic acid with increased cation-binding capacity (KULONEN 1952).

Summary

The effects of ascorbic acid on the composition of skin have been examined in non-scorbutic normal and oestradiol treated mice.

Mild degrees of non-respiratory acidosis were induced by the treatment. The results have therefore been compared with those of a previous study in which similar changes in corrected bicarbonate of plasma were induced by treatment with ammonium chloride

Treatment with ascorbic acid caused a significant fall in plasma chloride, whereas treatment with ammonium chloride had raised the concentration of chloride in plasma.

Treatment with ascorbic acid raised the hexosamine content and reduced the amount of hydroxyproline in the skin of normal mice.

When oestradiol treated mice were treated with ascorbic acid for 7 days, sodium in excess was deposited in the skin. In contrast with this, a mobilisation of sodium from the skin was observed after treatment with ammonium chloride.

The results have been interpreted in terms of a binding of ascorbate ions to insoluble colloids of the connective tissue.

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Analgesic Action and Brain and Plasma Levels of Morphine and *Codeine in Morphine Tolerant*, Codeine Tolerant and Non-Tolerant Rats

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In a literature review SHAUMANN (1957) reported codeine to possess ⅓ to 1/6 the same analgesic potency of morphine for the rat, as measured by the tail flick method. No mention was made, however of the route of administration or the time interval between the injections and the testing for the degree of analgesia. Our investigation was, therefore, designed to compare the relative potencies of morphine and codeine in non-tolerant and tolerant rats at two time intervals after subcutaneous or intraperitoneal administration of the drugs.

Included in the study is a comparison of the degree of analgesia with the amounts of the analgesic drugs in whole brains of the animals. It is recognized that such studies have special limitations, in so far as the precise localization of the analgesic receptors in the central nervous system is not known. Experimental evidence does not favour the idea that morphine analgesia is associated with remarkably high concentration of the drug in one or more distinct areas in the brain (MULÉ & WOODS 1962 MULÉ, WOODS & MELLETT 1962). The previous work with morphine (JÓHANNESSON & SCHOU 1963a, b) was extended to test whether equianalgesic doses of morphine and codeine given to rats by subcutaneous or intraperitoneal injection produce the same levels of the drugs in brain and plasma.

The hypothesis of SANFILIPPO (1948) that the analgesic action of codeine is due to the conversion to morphine *in vivo* was tested by JÓHANNESSON & SCHOU (1963a), who demonstrated that morphine can be found in the brain of rats given i. p. codeine. The amounts of morphine found in the brain of these rats were not significantly different from those found in

experiment 20 hours after the last daily dose of morphine or codeine. At 20 hours after receiving the last injections in the tolerance periods, their reaction times (no drug having been injected) were found to be in the same range as those of the non-tolerant rats. This was also true when morphine tolerant rats were taken into experiment 2 hours after the last dose in the tolerance period.

The influence of excitation seen in morphine tolerant rats soon after the last dose in the tolerance period (cf MARTIN *et al* 1963) on the tolerance to the analgesic effect of morphine was studied in morphine tolerant rats 2 hours after the last dose of morphine. The effect of tolerance to codeine analgesia on the lethal action of the drug was also investigated.

Comparisons of the degree of analgesia with the drug levels in brain and plasma. Morphine or codeine were administered to non-tolerant rats by s.c. and i p routes in amounts that statistically gave the same degree of analgesia when tested 30 minutes after the injections. The doses were 5 mg/kg of morphine or 60 mg/kg of codeine s.c. and 10 mg/kg of morphine or 30 mg/kg of codeine i p The animals were anesthetized with ether 27 minutes after the injections. About 1 minute later the abdomen was cut open and the aorta punctured and 6–10 ml of blood were withdrawn in 2–3 minutes. Then the total brain (including the brain stem and the cerebellum) was removed and stored deep-frozen until analyses for morphine and codeine were performed. The blood was centrifuged and the plasma stored frozen until it was analysed. For comparison, tolerant rats were also given these amounts of morphine and codeine and treated in the same way as the non-tolerant rats.

Morphine and codeine were determined in 0 7–1 0 ml of plasma and 4 measured amounts were analysed from each animal. The plasma samples were diluted to a volume of 3 ml. The total brains of the animals were homogenized with a Teflon pestle after adding a sufficient volume of 1 M NH_4OH solution to make a total volume of 10 ml. Two 3 ml portions were used from each brain each portion thus containing about 500 mg of brain tissue.

Determinations of morphine and codeine N-C^{14} Methyl labelled morphine and codeine were synthesized by a slight modification of the method of ANDERSON & WOODS (1959) The specific activity of the morphine and the codeine was about 1 mC/mM

The estimation of the radioactive drugs in biological materials is, with modification, that of MULÉ & WOODS 1962, MULÉ, WOODS & MELLETT 1962 and CHERNOV & WOODS 1964 Three ml portions of fluid or homogenate were transferred to 43 ml centrifuge tubes, containing 0.5 mg of non-labelled morphine or codeine carrier or both. Two ml of 40 ⁄ (w/v) dibasic potassium phosphate (anhydrous) plus 15 ml of ethylene chloride (Fisher Scientific reagent grade) containing 10 ⁄ (v/v) *n*-amyl alcohol (Matteson Coleman & Bell reagent grade) were added. The mixture was shaken for 30 minutes at 280–300 oscillations/min. in an International shaker machine and then centrifuged at 3000 r p.m. for 15 minutes. The upper aqueous layer was removed by aspiration and the organic phase washed by shaking the tubes for 30 seconds after adding 4 ml of 4 ⁄ (w/v) dibasic potassium phosphate. Then the tubes were again centrifuged at 3000 r p.m. for 15 minutes, and the aqueous phase was removed by aspiration.

Ten ml portions of the organic phases, containing morphine or codeine or both, were transferred to 20 ml scintillation counting vials and evaporated during the night on a Fisher slide-warmer at 55° The residue upon evaporation was dissolved in 0.8 ml *n*-amyl alcohol. Ten ml of a scintillating solution, containing 6 g of 2,5-diphenyloxazole and 200 mg of 1 4-bis-2-(5-phenyloxazolyl)-benzene in 2000 ml of analytical grade

toluene, were added, and its radioactivity was determined in a Nuclear-Chicago Model 720 Series Liquid Scintillation System. The samples were counted for 3 × 10 minutes. Controls for the background and controls with known amounts of labelled morphine and codeine were run concurrently to serve as check on the technique and performance of the counter

When the animals had received codeine, attempts were made to separate biotransformed morphine from codeine in brain and plasma. For this purpose one half of the samples analysed from each animal (one from brain and two from plasma) were shaken for 10 minutes with 4 ml of 0 1 N-NaOH after they had been washed with the 4% potassium phosphate buffer (see afore). By this procedure morphine is transferred as sodium phenolate to the aqueous phase, while codeine remains in the organic phase. The aqueous phase was removed by aspiration the organic phase was washed with 4% potassium phosphate buffer and centrifuged at 3000 r.p.m. for 15 minutes. Then the buffer was removed, and 10 ml portions of the organic phase were transferred to the counting vials and counted as usual. The differences in net counts per minute (net CPM) between the samples treated and the samples not treated with sodium hydroxide therefore indicated the amounts of morphine in the tissue.

Aqueous dilutions were made from the injection solutions. Portions (3 ml) of these dilutions containing 10–1000 ng of labelled morphine or 100–6000 ng of labelled codeine, mixed with the respective non-labelled drug, were taken through the analytical procedure. Known amounts were taken out of the aqueous dilutions and added to brain homogenates (10 / dilution) and plasma (10 / dilution) and analysed. A perfect linear relationship was always observed when net CPM values were plotted against the total amounts of the labelled drugs (correlation coefficient, = 1.0).

Several 0.1 ml portions of the aqueous dilutions were transferred to the counting vials and evaporated to dryness for 2 hours. The residue upon evaporation was dissolved and counted as described. A perfect linear relationship was observed when net CPM values were plotted against the total amounts of the labelled drugs. It was calculated that 1 ng of synthesized C^{14}-labelled morphine corresponded to 4.87 net CPM with limits of error of the mean (L.E.) ± 3.2 at P = 0.95 1 ng of C^{14}-labelled codeine corresponded to 4.56 net CPM ± 1 0 / (L.E.). These figures were taken as the 100 % values for morphine and codeine, respectively The percentage recoveries for morphine were 98.6 ± 3.5 / (L.E.) from water 93.2 ± 2.4 / (L.E.) from brain homogenates and 91.6 ± 2.6 / (L.E.) from plasma. The corresponding figures for the percentage recoveries of codeine ± L.E. were 98 7 ± 3.2 %, 96.5 ± 2.3 / and 96 1 ± 2.1 In experiments on drug distribution, the mixed drugs (labelled + non-labelled) were, as mentioned before, administered to the animals. Appropriate multiplication factors were therefore used to calculate the total amounts of the drugs in brain and plasma. The results (ng/g, ng/ml) refer to the total amounts of the "free" or unconjugated drugs only

Mixtures of the aqueous dilutions, containing known amounts of morphine and codeine, were taken through the analytical procedure. Known amounts of the drugs in aqueous mixtures were added to brain homogenates and plasma, which were analysed. Lower amounts than 5 ng of labelled morphine could not be separated from 5 ng or higher amounts of labelled codeine. Morphine could not be separated from codeine if the amounts were less than 3–4 / of those of codeine in the mixture. The greater the percentage of morphine in mixtures of the two drugs, the higher were the recoveries of morphine. The following percentage recoveries of morphine and codeine ±L.E. refer to experiments in which the amounts of labelled morphine were 4–15 of those of labelled codeine morphine 90.6 ± 11.0 from water 84.2 ± 10.2

from brain homogenates and 82.5 ± 12.6 / from plasma. Codeine 99 6 ± 1 6 / from water 101 / ± 1 6 / from brain homogenates and 99 6 ± 1 8 / from plasma.

In analysing brains from a few rats injected with morphine, 10–20 / quenching of the counts was observed. This was due to yellow colour extracted from the tissue. The quenching was corrected by adding internal standard of C^{14} The χ^2 test was performed as described by BURN *et al* (1950) Other statistical calculations were performed by the procedures of SAUNDERS & FLEMING (1957). In test of significance the null hypothesis was rejected if $p' < 0.05$

Results

Analgesimetry

1 *Non-Tolerant Rats* The results of the analgesic experiments with morphine and codeine after s c. or i p administration are summarized in fig. 1 & 2 The analgesic potency of morphine was about twofold greater after s c. than after i p injections. For codeine the reverse was true. Morphine is therefore 10–12 times more potent as an analgesic than codeine at the ED50 level if the drugs are given by the s.c. route, whereas i.p morphine exhibits only a threefold greater potency than codeine

The degree of analgesia was, except with subcutaneous codeine, generally greater 30 minutes than 60 minutes after the injections. Thus, except for s c. codeine, the lowest doses of the drugs produced analgesia in less than 10% of the animals 60 minutes after the injections.

As illustrated in fig. 1 & 2, pretreatment with reserpine 4½ hours before administration of the narcotic agent did not alter the analgesic effect of morphine or codeine In another experiment rats were administered reserpine (2 mg/kg) 16 hours before s.c injection of 60 mg/kg of codeine. Although these animals were highly sedated by the pretreatment with reserpine, they showed the same degree of analgesia as control rats at 30 and 60 minutes after the injection of codeine

2 *Tolerant Rats* The comparative analgesic effects of administering morphine and codeine by i p or s.c. injection to non tolerant morphine tolerant and codeine tolerant rats are shown in table 1 At 30 minutes after the injections, the morphine tolerant rats exhibited less analgesia after morphine or codeine than did the codeine tolerant rats However the latter animals did demonstrate a significantly lower analgesic effect by morphine or codeine than the non-tolerant rats At 60 minutes after the injection, either one or none of the morphine tolerant and codeine tolerant rats in each group was analgesic

Codeine Lethality The lethal dose of codeine by i p injection was

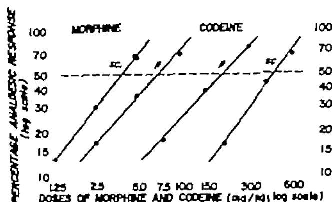


Fig. 1 Analgesia produced by morphine and codeine in non-tolerant rats by s.c. or i.p. injection in tests performed 30 minutes after injection. The response of rats pretreated with reserpine is also shown. *Abscissa*: doses of morphine and codeine, log. scale *Ordinate*: percentages of animals reacting analgesic (log. scale). The number of animals in each group was 22 26. a reserpine pretreated.

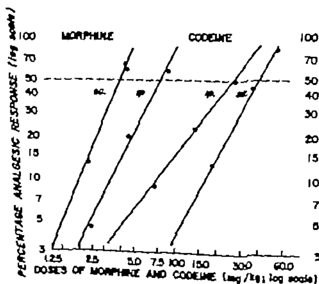


Fig 2 Analgesic effect of morphine and codeine 60 minutes after the injections. For explanations, see Fig. 1

from brain homogenates and 82.5 ± 12.6 / from plasma. Codeine 99 6 ± 1 6 / from water 101 / ± 1 6 / from brain homogenates and 99 6 ± 1 8 / from plasma.

In analysing brains from a few rats injected with morphine, 10–20 / quenching of the counts was observed. This was due to yellow colour extracted from the tissue. The quenching was corrected by adding internal standard of C^{14} The χ^2 test was performed as described by BURN *et al.* (1950). Other statistical calculations were performed by the procedures of SAUNDERS & FLEMMING (1957) In test of significance the null hypothesis was rejected if $p^1 < 0.05$

Results

Analgesimetry

1 *Non Tolerant Rats* The results of the analgesic experiments with morphine and codeine after s.c. or i.p administration are summarized in fig 1 & 2. The analgesic potency of morphine was about twofold greater after s c. than after i p injections. For codeine the reverse was true. Morphine is therefore 10–12 times more potent as an analgesic than codeine at the ED50 level if the drugs are given by the s c route whereas i p morphine exhibits only a threefold greater potency than codeine

The degree of analgesia was except with subcutaneous codeine, generally greater 30 minutes than 60 minutes after the injections Thus, except for s c. codeine, the lowest doses of the drugs produced analgesia in less than 10 / of the animals 60 minutes after the injections.

As illustrated in fig. 1 & 2, pretreatment with reserpine 4½ hours before administration of the narcotic agent did not alter the analgesic effect of morphine or codeine In another experiment, rats were administered reserpine (2 mg/kg) 16 hours before s.c injection of 60 mg/kg of codeine. Although these animals were highly sedated by the pretreatment with reserpine, they showed the same degree of analgesia as control rats at 30 and 60 minutes after the injection of codeine

2. *Tolerant Rats* The comparative analgesic effects of administering morphine and codeine by i p or s.c. injection to non tolerant, morphine tolerant and codeine tolerant rats are shown in table 1 At 30 minutes after the injections, the morphine tolerant rats exhibited less analgesia after morphine or codeine than did the codeine tolerant rats. However the latter animals did demonstrate a significantly lower analgesic effect by morphine or codeine than the non tolerant rats. At 60 minutes after the injection, either one or none of the morphine tolerant and codeine tolerant rats in each group was analgesic.

Codeine Lethality The lethal dose of codeine by i p. injection was

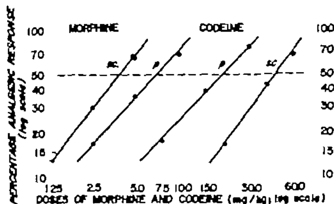


Fig. 1. Analgesia produced by morphine and codeine in non-tolerant rats by s.c. and i.p. injection in tests performed 30 minutes after injection. The response of rats pretreated with reserpine is also shown. *Abscissa*: doses of morphine and codeine, log. scale *Ordinate*: percentages of animals reacting analgesic (log. scale). The number of animals in each group was 23–26. ● reserpine pretreated.

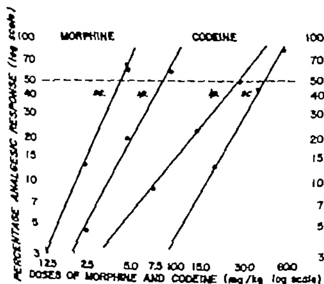


Fig. 2. Analgesic effect of morphine and codeine 60 minutes after the injections. For explanations, see Fig. 1

Table 1

Analgesic effects of morphine and codeine in non-tolerant, morphine tolerant and codeine tolerant rats at 30 and 60 minutes after the injections. The tolerant rats, except one group of morphine-tolerant rats, were taken into experiment 20 hours after the last dose in the tolerance period.

Drugs and Doses mg/kg	Non-tolerant)			Morphine tolerant			Codeine tolerant		
	Nr of Animals	Percentage analgesic response		Nr of Animals	Percentage analgesic response		Nr of Animals	Percentage analgesic response	
		30min.	60min.		30min.	60min.		30min.	60min.
Morphine 5.0 *sc*	23	65	65	20	0	0	22	14	5
Codeine 60.0 *sc*	22	68	82	18	0	0	23	30)	4
Codeine 30.0 *ip*	26	77	50	[illegible]0	10	5	14	[illegible]9)	7
Morphine 10.0 *ip*	[illegible]6	69	58	18	11	6			
Morphine[1]) 10 0 *ip*				16	0	0			

s.c. subcutaneous injections *i.p.* intraperitoneal injections.

[1]) These were taken into experiment 2 hours after the last daily dose of morphine.

) The same values as depicted in figs. 1 and 2. These doses were taken as equianalgesic at 30 minutes comparison of *morphine 5 0 s.c* and *codeine 30.0 i.p* gave a value of χ^2 lower than 0 4 In a parallel study with similar numbers of non-tolerant rats, these 4 doses produced analgesia in 70 ,, 76 / 77 and 71 of the animals at 30 minutes (unpublished results)

) Significantly lower degree of analgesia than in the non tolerant rats ($\chi^2 > 3.84$).

compared in non tolerant and codeine tolerant rats (table 2) Although only few animals were used, the result indicate, that codeine tolerance (to the analgesic effect) does not markedly alter the lethal action of codeine. All the animals that died, non tolerant and codeine tolerant, showed generalized convulsions. The minimal lethal dose of codeine is greater than 200 mg/kg if the drug is given by s c injections.

Table 2

Lethal effects of *i.p* codeine in codeine tolerant and non tolerant rats. The numbers of rats dying within 60 minutes as well as those of rats that survived this period are given.

Doses mg/kg	Non-tolerant			Codeine tolerant		
	Nr of Animals	Nr of Animals Dying	Nr of Animals Surviving	Nr of Animals	Nr of Animals Dying	Nr of Animals Surviving
125	10	4	6	10	7	3
150	10	7	3			

Table 3

Concentrations of morphine in brain (ng/g) and plasma (ng/ml) of 16 non-tolerant and 4 morphine tolerant rats at 30 minutes after injection. Eight of the non-tolerant rats received morphine by s.c. and 8 by i.p. injection. *The tolerant rats* were given the drug by i.p. injection. The results indicate the amounts of labelled as well as those of the non-labelled drug. The means are gi en at the bottom.

Non-tolerant 5 mg/kg s.c.		Non-tolerant 10 mg/kg i.p.		Tolerant Given 10 mg/kg i.p.	
Morphine in Plasma	Morphine in Brain	Morphine in Plasma	Morphine in Brain	Morphine in Plasma	Morphine in Brain
970	175	1020	200	495	160
1240	170	980	170	570	170
1180	235	815	185	540	165
1150	190	860	215	665	185
910	145	910	225		
840	195	570	200		
900		735	140		
645	145	805	130		
m: 982*	179	839*	183	568+	170

*) Not significantly different ($t < 2.15$).

+) Significantly lower than the values marked with * ($t > 2.23$).

Drug Concentrations in Brain and Plasma (tables 3 & 4). The morphine concentration in the brain of the non-tolerant rats was on an average *approximately 180 ng/g 30 minutes after s.c. injection of 5 mg/kg of* morphine and after i.p. injections of 10 mg/kg (these are equinalgesic doses by the specified routes), and not significantly lower in the brain of morphine tolerant rats given 10 mg/kg of i.p morphine. Plasma concentrations of morphine were approximately fivefold greater than the brain concentrations in the non-tolerant rats, after 5 mg/kg of s.c. morphine or 10 mg/kg i. p., but only about threefold greater in the morphine-tolerant rats.

In contrast to the results of experiments with morphine, the administration of codeine, in doses equianalgesic with those of morphine, gave 30 minutes after the injections a mean brain concentration of codeine of about 9000 ng/g after 60 mg/kg by the s.c. route and about 8000 ng/g after 30 mg/kg of codeine by the i.p route. In each instance the mean plasma concentration was approximately ½ the brain concentration. The s.c. injection of 60 mg/kg of codeine and i.p. injection of 30 mg/kg to codeine tolerant rats gave concentrations of codeine in brain, 30 minutes after administration, on an average of about 7000 ng/g and 11500 ng/g, respectively. Again, the mean concentration of codeine in plasma was about ½ *of that* in the brain.

Table 4

Concentrations of codeine in brain (ng/g) and plasma (ng/ml) of 16 non-tolerant and 18 *codeine tolerant* rats 30 minutes after they were given the drug by s.c. (8 & 8) or i.p injection (8 & 10) The amounts of morphine, formed from codeine *in vivo* are given for the plasma In brain morphine was not *detected* after injections of codeine. The results indicate the amounts of the labelled as well as those of the non-labelled drugs. The means are given at the bottom.

Non-tolerant rats						Codeine tolerant rats					
60 mg/kg s.c.			30 mg/kg i.p.			60 mg/kg s.c.			30 mg/kg i p.		
Codeine in Brain	Codeine in Plasma	Morphine in Plasma	Codeine in Brain	Codeine in Plasma	Morphine in Plasma	Codeine in Brain	Codeine in Plasma	Morphine in Plasma	Codeine in Brain	Codeine in Plasma	Morphine in Plasma
7285	3870	165	7970	6615	690	5730	3145	190	5325	2110	145
12575	6415	215	12735	4025	755	5570	2710	150	13565	5490	575
7850	4590	225	7315	3750	515	4140	3970	175	4160	2335	215
11225	5935	315	6106	2775	150	10060	4330	120	14253	5710	470
11860	5990	265	8180	3870	590	6700	4255	103	13205	5520	440
7345	3845	200	6360	2975	490	5850	3310	205	4885	1985	230
5860	2825	155	6915	5380	520	6975	3630	150	15955	7075	525
8260	3570	265	7240	3280	480	11140	5910	190	14913	6005	430
	–	–	–	–	–	–	–	–	14735	6380	460
		–	–	–	–	–	–	–	14560	6520	480
m 9033[1,3]	4630[2,4]	226	7877[1,5]	4084[2,6]	524	7023[3]	3908[4]	161	11498[5]	4913[6]	397

1.1 2.2 3.3 4.4 not significantly different (t < 2.15)
3.3 .4 Not significantly different (t < 2.10).
) Significantly different (t > 2.15)
) Significantly different (t > 2.10)

The codeine/morphine ratio in the plasma of non-tolerant rats was approximately 20 to 1 after s.c. injection of 60 mg/kg of codeine, but only 8 to 1 after i.p. administration of 30 mg/kg of codeine. Thus, although the i.p. dose was half that of the s.c., the former gave on an average more than twofold a higher concentration of biotransformed morphine in plasma. On the basis of the plasma concentrations of morphine, the conversion of codeine to morphine was less in the codeine tolerant rats.

The presence of morphine, biotransformed from codeine, could no tbe demonstrated in brain tissue of rats after either s.c. i.p. injections of codeine. Since the plasma/brain ratio for morphine was about 5 to 1 in non-tolerant rats given morphine (table 3), the same ratio should hold for morphine formed from codeine *in vivo*. The predicted brain concentration of morphine in the non-tolerant rats, given 60 mg/kg of s.c. codeine or 30 mg/kg by the i.p. route, would therefore be about 45 ng/g or 105 ng/g, respectively. It is, as mentioned before (cf. methods), not possible to quantitate so small amounts of morphine in presence of the large quantities of codeine found in the brain.

Discussion and Conclusions

Apart from analgesia due to s.c. codeine, the degree of analgesia produced by codeine and morphine in non-tolerant rats was less at 60 minutes than at 30 minutes after the injections (fig. 1 & 2). This difference was particularly striking in the tolerant rats (table 1), an observation in agreement with the classical view that tolerance to analgesia is characterized by shorter duration of action as well as by lower intensity of effect (ROSSBACH 1880).

Morphine was a more potent analgesic than codeine, as could be expected, but the relative potencies varied as much as fourfold, depending upon the route of administration, whether subcutaneous or intraperitoneal. When codeine and morphine are being compared, the *route of administration* must therefore be stated.

Equianalgesic doses of morphine given to non-tolerant rats (5 mg/kg s.c. and 10 mg/kg i.p.) were associated with the same concentrations of the drug in brain and plasma (table 3). The remarkably high analgesic potency of s.c. morphine therefore indicates a relatively greater or faster rate of absorption of "free" morphine or a slower rate of detoxication than when the drug is injected by the i.p. route. Only the "free" or nonconjugated drugs were determined in the present study since the quantity of biological material available did not permit the further quantitative determination of "bound" or conjugated drug.

Equianalgesic doses of codeine (60 mg/kg by the s.c. and 30 mg/kg by

the i p route gave closely similar concentrations of the drug in brain and plasma of non tolerant rats (table 4) The equianalgesic doses of codeine were also equianalgesic with those of morphine mentioned above (cf table 1) On the basis of comparative concentrations in brain, it appears that morphine, molecule for molecule, is about 50 times as potent an analgesic agent as codeine (cf tables 3 & 4) In her experiments on the administration of analgesics into the cerebral ventricles of mice, ADLER (1963) found morphine to be about 100 times as potent an analgesic as codeine Brain tissue possesses little, if any ability to convert codeine to morphine (ELISON & ELLIOTT 1963 JÓHANNESSON & ROGERS unpublished results) Therefore, the question must be faced whether the relatively high analgesic potency of codeine observed after peripheral administration of the drug in the investigation recorded here, is due to biotransformed morphine gaining access to the brain (see below) It should here be mentioned that normorphine and norcodeine if present in brain and plasma of the animals, would not have been determined by our technique

Although biotransformed morphine was found in the plasma of all rats given codeine, morphine could not be detected in brain tissue because of analytical difficulties associated with the presence of large quantities of codeine The concentrations of biotransformed morphine in brain can, however be predicted to be approximately 45 and 105 ng/g after s.c injection of 60 mg/kg and i.p administration of 30 mg/kg of codeine, respectively (cf results) Whether or not these predicted or perhaps even higher amounts of biotransformed morphine were in fact present in the brain of the rats given codeine is conjectural, but certainly compatible with the results of an earlier investigation (JÓHANNESSON & SCHOU 1963a)

The enhanced analgesic effect of i p codeine, as compared with s.c. could result, *a priori* from higher (predicted) amounts of biotransformed morphine in the brain. This view is supported by the observation that simultaneous s.c administration of morphine and codeine to rats results in either an additive or even a potentiated analgesic effect (unpublished results) A reasonable assumption would then be that when codeine, and the morphine derived from it, are both found in brain a similar additive or potentiated action would result. The enhanced biological activity of codeine when given by the i p. route may on the other hand, result from a more rapid and more complete absorption than when the drug is injected by the s.c. route. The greater persistence of the analgesic effect of s.c. codeine is compatible with this idea It should also be emphasized that the rate of conjugation of codeine is extremely low in the rat (YEH personal communication) a phenomenon that must be advantageous for

the biological activity of the drug when it is given by i.p. injection. Nalorphine, moreover is even a more potent antagonist to the analgesic effect of codeine than to that of morphine (JÓHANNESSON, unpublished results). Therefore, although the morphine formed from codeine *in vivo* may contribute somewhat to the analgesic action of codeine, especially after i.p. administration, the results also suggest that codeine possesses an analgesic effect in its own right.

In vitro experiments have shown that liver microsomes from non-tolerant rats convert significantly greater amounts of codeine to morphine than liver microsomes from codeine tolerant animals (JÓHANNESSON & ROGERS, unpublished results). The *in vivo* experiments reported here confirm the *in vitro* studies. BOUESQUET, RUPE & MIYA (1964) have reported that chronic pretreatment with morphine produces a (non-specific) inhibition of metabolic pathways in the rat. Investigations are now in progress in this laboratory to determine the possible occurrence of a similar inhibition of drug metabolism induced by chronic pretreatment with codeine.

The codeine tolerant rats were less tolerant to the analgesic effect of codeine 30 minutes after the injections than were the morphine tolerant rats to morphine. Significant cross-tolerance between the two drugs was also apparent. Tolerance to the lethal effect of morphine in rats may develop concomitantly with tolerance to the analgesic action (JÓHANNESSON 1962, JÓHANNESSON & SCHOU 1963b, JÓHANNESSON & LONG 1964). This is apparently not true for codeine (table 2). Usually high doses of morphine produce death in rats in flaccid paralysis, although convulsions may precede death. However, death after codeine is invariably associated with convulsions.

Plasma levels of morphine were significantly lower in the morphine tolerant than in the non-tolerant rats 30 minutes after i.p. injection of 10 mg/kg of the drug, although the brain levels were the same in both groups of rats (table 3). Therefore, low or absent analgesic effect of morphine in the tolerant rats cannot be due to a lower level in or absence of the drug from brain, an observation that confirms the previous results of JÓHANNESSON & SCHOU (1963b). Neither is tolerance to codeine related to lower concentrations of the drug in brain (table 4). The lower plasma levels of morphine tolerant animals has no ready explanation on the basis of this investigation.

The administration of morphine to rats made tolerant to the drug by chronic pretreatment for several weeks produces an excitatory state, which is most prominent about two hours after the injection, a fact that has been described by MARTIN *et al.* (1963) and others, and is apparently paralleled by a significant increase in brain catecholamines (SLOAN *et al.* 1963). After 24 hours the brain levels of catecholamines returned to

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normal (SLOAN *et al*) and the levels of 5-hydroxytryptamine in brain were unchanged during addiction and abstinence. In the experiments recorded here the morphine tolerant rats were in an exhaustion like state at 20 hours after the last dose of morphine in the tolerance period. The animals showed at this time a small but significant analgesic effect at 30 minutes after 10 mg/kg of i.p morphine No analgesic effect was seen when the dose of i p morphine was given to the animals two hours after the last scheduled dose during the addiction cycle. It would nevertheless seem unlikely that tolerance to the analgesic action of morphine in the rat is related to the early signs of stimulation or depression in the abstinence period or to the levels of catecholamines in the brain particularly since pretreatment with reserpine has no effect on the analgesic effect of morphine or codeine (fig. 1 & 2, and results) or in a previous investigation (JÓHANNESSON & SCHOU 1963b) The apparently heterogenous nature of the abstinence symptoms (cf HUIDOBRO *et al* 1963a, b) may also indicate that the signs of morphine abstinence are not closely related to its analgesic effect.

Summary

At the ED50 level morphine is 10–12 times more potent as an analgesic than codeine if the drugs are given by s c. injection whereas after i.p injections morphine exhibits a potency only three times that of codeine. Except with s c. codeine, the degree of analgesia tended to be lower at 60 than at 30 minutes after injection

Equianalgesic doses of morphine given to non tolerant rats, whether s c. or i p resulted in the same concentrations of the drug in brain and plasma. Likewise, nearly identical amounts of codeine were found in brain and plasma when equianalgesic doses of the drug were given to non tolerant rats by s.c or i.p injection

Morphine, formed from codeine *in vivo* was found in the plasma of all rats given codeine. Biotransformed morphine could not be demonstrated in the brain of these rats because of analytical difficulties However most of the available information supports the idea that the analgesia produced by codeine is not entirely due to biotransformed morphine in the brain

If tested 30 minutes after the injections, codeine tolerant rats were less tolerant to the analgesic effect of morphine and codeine than were morphine tolerant rats. The experimental findings suggest that tolerance to codeine analgesia does not involve tolerance to codeine lethality

Pretreatment with reserpine did not affect the analgesic effect of morphine or codeine. Tolerance to the analgesic effect of morphine is apparently not related to brain levels of catecholamines.

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Alkalosis During the Disulfiram-Ethanol Reaction in Unanaesthetized Rabbits

By

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HALD JACOBSEN & LARSEN (1948) were the first to demonstrate that ethanol elicits a characteristic reaction, including hypotension, hyperventilation and other symptoms, in human subjects pretreated with disulfiram. PERMAN (1962) showed that ethanol could produce a similar symptom complex in rabbits pretreated with disulfiram, thus providing a convenient method for experimental studies of the disulfiram-ethanol reaction One feature of the reaction is a significant fall in plasma potassium as measured in unanaesthetized rabbits by BRUNDIN GREEN & PERMAN (1962) The mechanism of this electrolyte disturbance is not known. PERMAN (1962) described a slight increase in arterial blood pH during the initial phase of the disulfiram-ethanol reaction in urethane-anaesthetized rabbits. However the marked hypokalaemia found during the reaction in awake rabbits could not be due to this slight change in the acid-base balance. In humans RABY (1954) found an alkalosis during the reaction. In the study here recorded, the arterial blood pH was followed by a new method in awake rabbits during the disulfiram-ethanol reaction. The arterial blood pressure was also recorded, to define the reaction.

Methods

Male albino rabbits (2.5–4 kg) were used. Under local anaesthesia polyethylene catheters (INTRAMEDIC PE 50) were inserted into the central arteries and marginal veins of both ears. To prevent coagulation, i. heparin (4000 IE) was given. The arterial catheter from one ear was connected with the reciprocal vein via a glass chamber containing a pH electrode (RADIOMETER GK 264), attached to a pH-meter (RADIOMETER PHM 22). To prevent blood from entering the calomel part of the electrode, the arrangement described by BYDEMAN (1964) was used. The upper part of the electrode was connected with a branch from the arterial catheter by a saline-filled plastic tube, in whose bottom a thin latex finger stall transmitted the pressure f om the saline of the tube to the potassium chloride of the electrode. The

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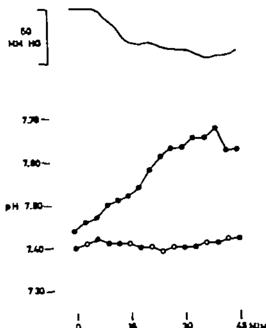


Fig. 2. Arterial blood pressure and arterial blood pH during the disulfiram-ethanol reaction in rabbits. Abscissa: time in min after beginning ethanol infusion. Upper ordinate: mean fall in arterial blood pressure after ethanol infusion into disulfiram pretreated rabbits, lower ordinate: mean arterial blood pH after ethanol infusion into rabbits not pretreated (○——○) and rabbits pretreated with disulfiram (●——●).

Discussion

The marked alkalosis that occurs during the disulfiram-ethanol reaction is of a magnitude sufficient to cause general electrolyte disturbances. The earlier described hypokalaemia during the reaction could therefore be secondary to the alkalosis. The somewhat slight increase of arterial blood pH found in urethane-anaesthetized rabbits (PERMAN 1962) was not confirmed by these results. Possibly the general anaesthesia, which can sometimes cause an acidosis, exerted a disturbing effect on the acid-base balance. About the origin of this alkalosis nothing is known. *Hyperventilation*, which is a classical feature of the disulfiram-ethanol reaction, indicates that the origin alkalosis is respiratory which would be in accordance with RABYS (1954) findings in man. Probably other electrolyte disturbances occur during the disulfiram-ethanol reaction, but so far this matter has not been studied.

Summary

By a new method arterial blood pH was followed in awake rabbits. During the induced disulfiram-ethanol reaction they showed a marked

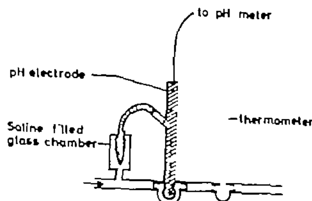


Fig. 1 Arrangement for measuring blood pH in awake rabbits. Arrow indicates direction of blood stream.

blood streamed around the electrode, through an outflow tube into another glass chamber containing a thermometer and then into the venous catheter as seen in fig. 1 Through this extracorporeal loop containing 2.3 ml a blood flow of 6-10 ml/min. was spontaneously maintained during the experiments. The blood temperature in the loop was lowered to 30°C. The primary pH values were registered every third minute and corrected to 39°C by the procedure of ROSENTHAL (1948) From the other ear artery the blood pressure was recorded by the method of PERMAN (1962).

Ethanol (4 4 mmoles/kg. i.v) was infused during 5–10 min. in 3 rabbits pretreated with 1 + 1 g disulfiram by stomach tube 24 and 2 hours before the experiments the same dose of ethanol was infused in 2 control rabbits not so pretreated with disulfiram.

Results

Arterial blood pH showed a marked increase after ethanol administration to those rabbits pretreated with disulfiram. The maximal alkalosis occurred about 40 min. after the ethanol infusion began (table 1) There was also as expected, a fall in arterial blood pressure in these animals (fig. 2.) No overt changes in arterial blood pH or in the blood pressure occurred in the control group

Table 1

Changes in arterial blood pH after ethanol administration to rabbits pretreated with disulfiram and to controls receiving the same dose of ethanol.

	Number of experiments	Initial values	40 min after ethan 1
Controls	2	7 40 ± 0.02)	7 41 ± 0 03
Pretreated with disulfiram	3	7 44 ± 0.04	7 68 ± 0.03

) Mean ± standard error of mean.

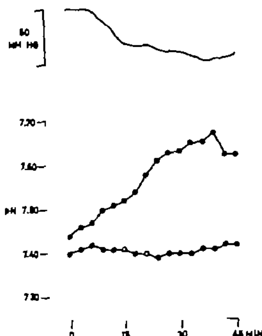


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Acknowledgements.

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